Susceptibility of Interleukin 1-beta (IL-1β) gene polymorphisms associated with Rheumatoid arthritis

Rabia Nasir

Regn. No.00000361136

MS Healthcare Biotechnology

Supervised by

Prof. Dr. Peter John

Atta-ur-Rahman School of Applied Biosciences (ASAB),

National University of Sciences and Technology (NUST), Islamabad

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By

Rabia Nasir

(Fall 2023-MS HCB- 00000361136)

Supervised by: **Prof. Dr. Peter John**

Atta-ur-Rahman School of Applied Biosciences (ASAB),

National University of Sciences and Technology (NUST), Islamabad, Pakistan

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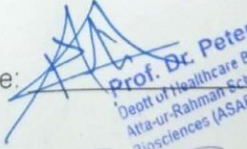1.   Name: Prof. Dr. Amir Ali Abbasi   Signature: _____

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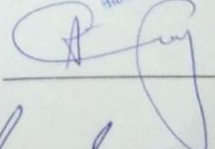
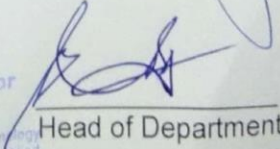Supervisor's name: Prof. Dr. Peter John   Signature: _____

Prof. Dr. Peter John
Deptt of Healthcare Biotechnology
Atta-ur-Rahman School of Applied
Biosciences (ASAB), NUST Islamabad

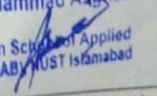Co- Supervisor's name: Prof. Dr. Attya Bhatti   Signature: _____

Date: 21.8.2023

Dr. Sobia Manzoor
Tenured Professor
Head of Department (HoD)
Deptt of Healthcare Biotechnology
Atta-ur-Rahman School of Applied
Biosciences (ASAB), NUST-Islamabad

_____ Head of Department

COUNTERSINGED

Prof. Dr. Muhammad Asghar
Principal
Atta-ur-Rahman School of Applied
Biosciences (ASAB) NUST Islamabad

Date: 21/8/23

_____ Dean/Principal

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Certified that final copy of MS thesis entitled "**Susceptibility of Interleukin 1-beta (IL-1β) gene polymorphisms associated with Rheumatoid arthritis**" submitted by Rabia Nasir (Registration # 00000361136), of Atta-ur-Rahman School of Applied Biosciences (ASAB), has been verified by undersigned, found that complete in all respects as per NUST regulations, and is accepted as fulfillment for the award of MS degree.

Supervisor: _____

Prof. Dr. Peter John

Prof. Dr. Peter John
Deptt of Healthcare Biotechnology
Atta-ur-Rahman School of Applied
Biosciences (ASAB), NUST Islamabad

Atta ur Rahman School of Applied Biosciences,

National University of Sciences and Technology.

Head of Department: _____ 17.8.2023

Prof. Dr. Sobia Manzoor

Dr. Sobia Manzoor

Atta ur Rahman School of Applied Biosciences,

National University of Sciences and Technology.

Principal: _____

Prof. Dr. Muhammad Asghar
Principal
Atta-ur-Rahman School of Applied
Biosciences (ASAB), NUST Islamabad

Prof. Dr. Muhammad Asghar

Atta ur Rahman School of Applied Biosciences,

National University of Sciences and Technology.

Dated: ___21/8/23___

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Rabia.

Rabia Nasir

Prof. Dr. Peter John
Deptt. of Healthcare Biotechnology
Atta-ur-Rahman School of Applied
Bioscience. (ASAB), NUST Islamabad

Supervisor: **Prof. Dr. Peter John**

Healthcare Biotechnology,

ASAB, NUST

DECLARATION

I, Rabia Nasir declare that this thesis titled "Susceptibility of Interleukin 1-beta (IL-1β) gene polymorphisms associated with Rheumatoid arthritis" and the work presented in it are my own and has been generated by me because of my own original research. I confirm that:

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2. Where I have consulted the published work of others, this is always clearly attributed. Where I have quoted from the work of others, the source is always given. Except for such quotations, this thesis is entirely my own work.

3. I have acknowledged all main sources of help.

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Rabia Nasir

Fall 2023-MS HCB-00000361136

DEDICATED TO

my wonderful parents, Muhammad Nasir Khan and Farkhanda Naz
for always loving & supporting me & for raising me to believe that
anything is possible

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Table of Contents

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

Rheumatoid arthritis is a progressive and chronic autoimmune disease which affects all racial and ethnic groups. The incidence or prevalence of rheumatoid arthritis varies across different geographic regions of the world and across different time periods. Globally it affects almost 0.5-1.0 per cent of the general population. In Pakistan it affects 0.3-0.9 per cent of the population. The disease can cause inflammation of one or more joints of the body. Several cytokines regulate key inflammatory pathways that are involved in the progression of disease. Interleukin-1 beta (IL-1β) is a crucial cytokine for the development of rheumatoid arthritis, which is secreted by synovial macrophages and is involved in the upregulation of inflammatory cells in the synovium, differentiation of osteoclast progenitors into preosteoclasts and regulation of the levels of other immunoregulatory molecule. Rheumatoid arthritis may be caused by genetic variations found in the IL-1β gene's regulatory regions. The important role being played by IL-1β signifies the candidacy of this gene for disease pathogenesis. In this study, the association of four SNPs rs2853550 (SNP-1, intergenic variant) and rs1143643 (SNP-2, intronic variant) of IL-1β gene and rs16944 (SNP-3, intergenic variant), rs3136558 (SNP-4 intronic variant) was studied in 100 rheumatoid arthritis patients from Pakistani population. These polymorphisms were examined using allele-specific PCR, and the data were statistically examined for any evidence of a connection between these polymorphisms and Rheumatoid arthritis. According to the findings of our study, SNP-1 and SNP-2 of the IL-1β gene were strongly related to Rheumatoid arthritis in our patients, however SNP-3 and SNP-4 were not. Other single nucleotide polymorphisms (SNPs) located in the regulatory and untranslated regions of the IL-1β gene may have significant association towards Rheumatoid arthritis, for which large scale data is required. However, more molecules implicated in the inflammatory pathway of rheumatoid arthritis need to be investigated for the genetic association with disease pathogenesis.

CHAPTER #1   INTRODUCTION

1.1 Rheumatoid arthritis

1.1.1 Introduction

Rheumatoid arthritis (RA) is a systemic and progressive autoimmune joint condition distinguished by a specific pattern of bone and joint degeneration (Choy, 2012). Several patient subgroups can be defined in RA as a systemic illness, depending on the presence or absence of extra-articular symptoms (Guo et al., 2018). For instance, the coexistence of anti-cyclic citrullinated peptide antibody (ACPA) and Rheumatoid factor (RF) distinguishes two significant patient categories (Tobón et al., 2010a).

Pain, stiffness, and swelling of the joints, as well as possible cartilage and bone degeneration that may lead to loss of joint function, are the primary symptoms of RA (van Delft & Huizinga, 2020). Rheumatoid arthritis is a long-lasting, systemic, an uncomfortable joint ailment that harms bone and cartilage and causes a wide range of extra-articular symptoms (Laska et al., 2014).

The cause of RA is unknown. It has become known in recent years that different factors like genetic and epigenetic factors, involved in the development of RA as well as environment also play a substantial role. (E. Liu & Perl, 2019). Joint pain, edema, and subsequent bone and cartilage degeneration are all symptoms of inflammation as systemic manifestations brought on by inflammatory cytokines and arachidonic acid metabolite (Scherer et al., 2020).

RA has a complex etiology like many autoimmune disorders. Familial clustering and monozygotic twin studies have genetic risk, with genetic predisposition accounting for 50% of the risk for RA (Michaud & Wolfe, 2007). Human leukocyte antigen-DR45 and DRB1, and several alleles referred to as the common epitope, have genetic correlations for RA (Magyari et al., 2014).

RA is characterized by inflammatory processes that cause the development of synovial cells in joints. Later pannus growth led to underlying cartilage degradation (Komatsu & Takayanagi, 2018). Interleukin-6 and tumor necrosis factor (TNF), these two proinflammatory cytokines that are produced in excess, accelerate the destructive process (Wasserman, 2011; Fiorella et al., 2022).

Human chromosome 2's long arm contains IL-1, which is grouped with IL1α and IL1β. It has been shown that an 86-bp tandem with a variable copy number causes a polymorphism in IL1RN intron

2 (Altomonte et al., 1992). Recent research in families with multiplex RA has suggested a connection between the IL-1 cluster genes and erosive RA (Carreira et al., 2005).

Synovial joints are first affected by rheumatoid arthritis (RA), a systemic autoimmune disease, before other organs including the cardiovascular or respiratory systems. When adaptive immune responses to autoantigens are triggered, dysregulated immunological homeostasis results, which is how autoimmune disease occurs (Alivernini et al., 2020). About 5% of the world's population suffers from one of the diverse groups of poorly understood diseases known as autoimmune diseases, which cause morbidity and mortality (Hejrati et al., 2021).

1.1.2 Epidemiology

The worldwide prevalence of RA is approximately 0.5-1%. Although it can happen at any age, the peak occurrence is at the age of 50, and it affects at least twice as many women as males (Kozłowska et al., 2022) It appears that the prevalence of RA may have increased after 1995. It is possible to hypothesize that this change may be explained by variations in environmental factors, but it is challenging to pinpoint a single offender (van der Woude & van der Helm-van Mil, 2018).

A meta-analysis of 67 RA-cohort recurrence and prevalence studies from 41 nations conducted in 2021 found that the prevalence for the years 1986–2014 was 0.46%. Even though this estimate is nearly twice as high as the GBD study's 2017 estimate of a global prevalence of 0.27 percent (Alamanos & Drosos, 2005)

According to the 2017 GBD data, the frequency of RA is highest in industrialized countries, followed by India and South American countries. The prevalence of RA also appears to be lower in rural than in metropolitan areas, while the published data are divided on this matter (Finckh et al., 2022). Another study conducted in Pakistan shows that the prevalence of RA is 3 times more in females than males as shown in Fig 1 (Rehan et al., 2015).
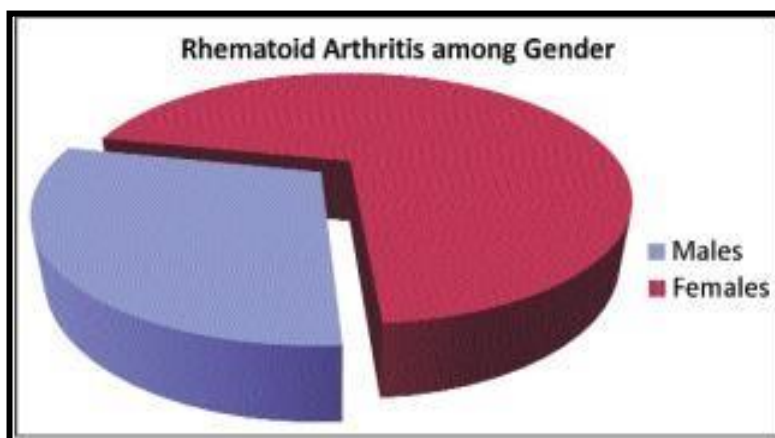
Fig 1.1.1: Rheumatoid arthritis prevalence with respect to gender

(Shamim R, Jan MD, Zafar U. Prevalence of rheumatoid arthritis in population with arthralgia presenting to a tertiary care hospital. J Pak Med Assoc. 2015 Nov;65(11):1202-5. PMID: 26564293)

According to reports, it affects 0.1% to 2.0% of people worldwide. Despite recent therapeutic advancements, the etiology of RA is still poorly understood, and there is no recognized cure. According to self-reported data from the NHS conducted in 2014–2015, Australia has the highest prevalence of RA (2% of the population) worldwide (Conforti et al., 2021). To alleviate the burden of this disease and to inform health policy, an accurate assessment of the prevalence of RA is necessary. It will also assist in determining the disorder and burden of care for RA patients (Almutairi et al., 2021).
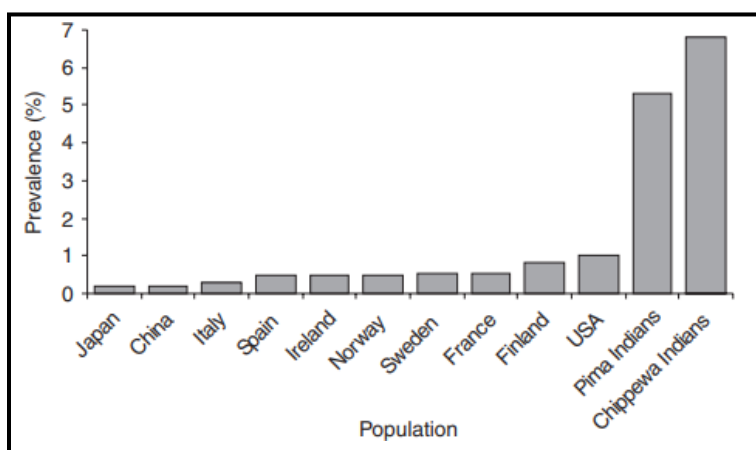
Fig 1.1.2: Prevalence of Rheumatoid Arthritis worldwide (Alan J Silman et al., 2002).

A study examined the prevalence of rheumatic symptoms among 1997 residents of northern Pakistan, equally distributed among wealthy urban, and poor rural communities. The most severe occurrence (8/1,000) was found in a wealthy metropolitan neighborhood (Ferucci et al., 2005). Both affluent and impoverished regions of southern Pakistan were researched regarding rheumatoid arthritis. Unexpectedly, older men were more likely than women to have rheumatoid arthritis, and the M: F ratio was 1:1 (Akhter et al., 2011).

Another study reported that RA is thought to be common in poorer nations. The prevalence of RA in India (0.75%) is comparable to that in the white population of Manchester6 (0.8%) (Macgregor et al., 1994), studies from Africa, Indonesia, and Nigeria all showed lower prevalence rates than those reported from the western nations. According to estimates, Karachi's urban population has a 0.142% prevalence of RA, compared to an estimated 0.55% in the north (Syed et al., 2011).

1.1.3 Pathogenesis of RA

A dysregulated immune response that causes increasing synovial inflammation and joint degeneration are thought to be the root cause of RA (Schiff, 2000). Even though T-cell involvement has been significantly indicated, the exact causes of RA's onset are still unknown. A result that suggests the process is mediated by T cells in the presence of CD4 T cells infiltrating the synovium in a characteristic manner (Dinarello, 2018).

Patients have been reported to have high levels of macrophage migration inhibitory factor and T-cell-generated chemokine-related cytokines. These natural compounds encourage cell growth, the expression of cellular adhesion molecules, metalloproteinases, and other inflammatory cytokines, and prostaglandin synthesis in synovial tissues, all of which may contribute to the pathogenesis of RA (Sayah & English, 2005).

Post-translational modification (PTM) of proteins, such as citrullination, carbamylation, and glycation, is one of the main processes that result in the production of autoantibodies (Mastrangelo et al., 2015). Most frequently, PTM produces changed amino acids with various immunological characteristics (Kolarz et al., 2021).

As the first class of autoantibodies discovered in Rheumatoid arthritis, RFs are the antibodies that recognize the Fc-tail region of immunoglobulin (Ig)-Gs, were used in 1987 ACR categorization

criteria for RA etiology. Studies have shown that a variety of isotypes, including IgM, IgG, and IgA, are used in the RF response (Liu et al., 2022).

The most extensively researched RFs are RF IgM and RF IgA since RF IgG is technically challenging to detect. Moreover, RF IgA autoantibodies have been proven to be crucial in clinical characteristic of RA (Dinarello, 2018b). ACPAs are another well-known autoantibody in RA. Citrullination is a recognized post-translational modification (PTM) due to ACPAs. Citrullination process is the enzymatic process of converting arginine to citrulline via peptidyl arginine deiminase (PAD) (van Delft & Huizinga, 2020).

Synovial hyperplasia is a main feature of RA and the main contributor to the development of an invasive and painful pannus. In RA, the normally composed of 1-3 cell layers synovial lining is notably thicker (Otero & Goldring, 2007). This is brought on by the invasion of cells resembling macrophages and the growth of local synovial fibroblasts (Kemble & Croft, 2021). The degree of synovial hyperplasia matches with the severity of cartilage erosions that causes inflammatory pannus forming and infiltrating joint cartilage, while osteoclast activation lads to parallel bone damage (Weyand & Goronzy, 2021). The synoviocytes in this region secrete large amounts of matrix-degrading enzymes such collagenase, stromelysin, and gelatinase (Scherer et al., 2020).

1.1.4 Role of autoimmunity in RA

IL-1β has a significant part in the development of pannus and inflammation in synovium. The expression of cell-adhesion molecules (like PGE2 and nitric oxide), other cytokines, chemokines, and chemokine receptors. Inflammation is increased by IL-1β through the stimulation of cyclo-oxygenase type 2 and inducible nitric oxide synthase. Large inflammatory inflammation is explained by high prostaglandins and other proinflammatory mediators (Tracy et al., 2017).

As a result, IL-1β frequently detects some discomfort, bruising, and tenderness in the rheumatoid joint. Because IL-β1 and TNF stimulate the expression of adhesion molecules occurs in the endothelium of high endothelial venules, inflammatory cells can penetrate the joint area. Furthermore, IL-1β and TNF increase the generation of collagenase from chondrocytes and activate osteoclasts in the bone as shown in fig 1.5 (Dayer, 2003).
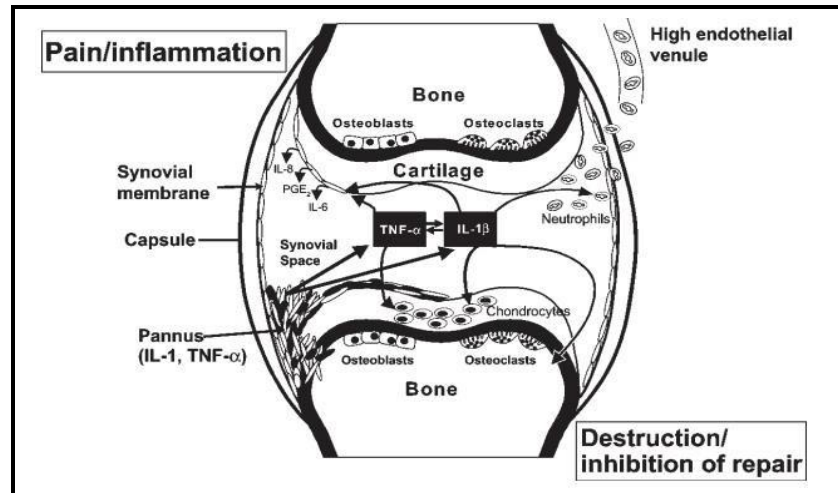
Fig 1.1.3: Role of Interleukin-1β in RA pathophysiology

TNF, a constituent of the TNF family, and B cell activating factor (BAFF), which encourages the growth of B cells and assists in the creation of synovial joint autoantibodies., are just two of the chemicals that synovial joint neutrophils produce (Moelants et al., 2013). Neutrophils can also produce the TNF superfamily member receptor activator of nuclear factor kappa B (NF-kB) ligand (RANKL), which is crucial for osteoclast formation and bone erosions in RA (O'Neil & Kaplan, 2019).
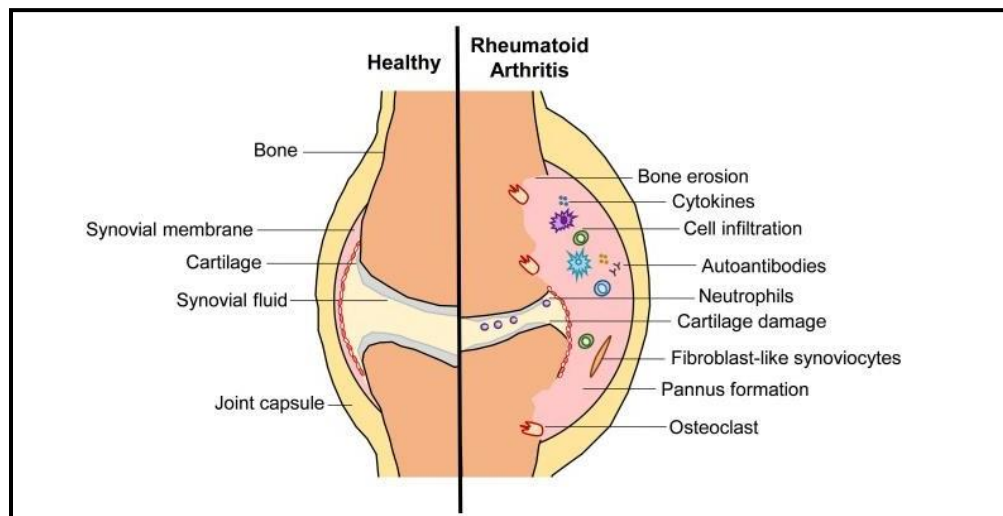
Fig 1.1.4: **Comparison of a healthy and a Rheumatoid arthritis joint**. A healthy joint can be seen on the left, whereas a rheumatic joint can be seen on the right. Due to the growth of synoviocytes that resemble fibroblasts and the invasion of peripheral blood cells in established RA, the inflammatory synovial membrane develops a pannus. These cells are extremely active,

generating inflammatory mediators and autoantibodies that feed the inflammatory process inside the joint. Osteoclast-mediated bone erosion and cartilage degradation are also present, which together cause the pannus tissue to invade and irreversibly distort that joint (Unterberger et al., 2021).

The most common inflammatory chronic systemic illness, rheumatoid arthritis (RA), is due to widespread synovitis, an immunological reaction that causes cartilage and bone erosion, and joint destruction as a result (Karami et al., 2019). The initial step towards the onset of autoimmune phenomena differentiation and bone erosions in RA is the loss of immune tolerance to self-antigens. Similarly, ACPA has been linked to higher mortality, disability, radiological disease progression, and severity of the disease in RA (Conigliaro et al., 2016)

The progression of bone degradation with RA is formed when early in the preclinical stage of RA, plasma cells start to make ACPA. ACPA can increase osteoclast differentiation and contribute to early bone loss (Derksen et al., 2017a). These early alterations could start in the bone marrow close to the joint. At the beginning of a clinical illness, synovitis causes the release of cytokines, which promote RANKL expression and drive osteoclastogenesis by stimulating bone degradation. Large bone erosions loaded with inflammatory, synovial-generated pannus tissue are signs of established RA (Volkov et al., 2020). The contact between inflammatory synovial tissue and the periarticular bone surface involves osteoclasts as shown in Fig 2.
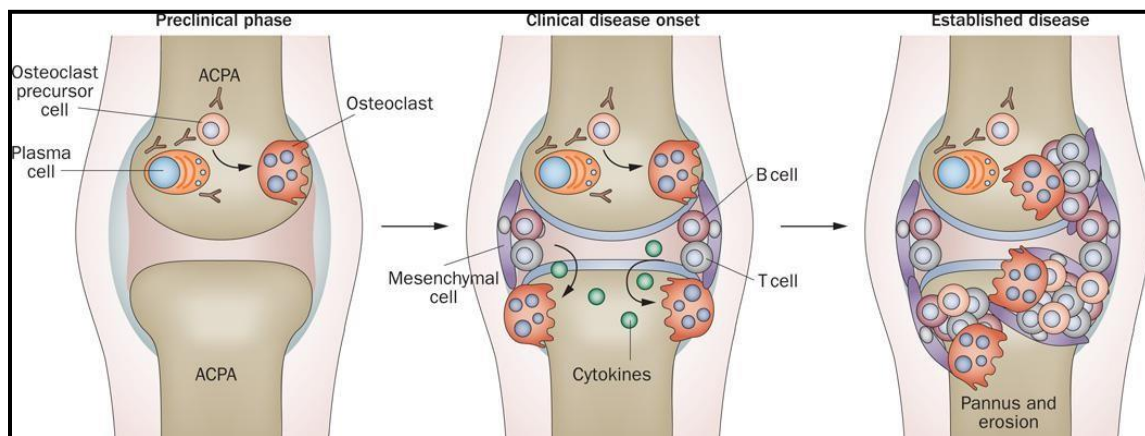
Fig 1.1.5: **Formation of bone erosion in RA**. Starting from a preclinical phase which involves osteoclast cells and plasma cells, then the onset of the disease which involved the role of B cells,

T cells, and mesenchymal cells, ultimately leading to established RA with pannus formation (Schett & Gravallese, 2012).

The JAK family has subtypes, including JAK1, JAK2, JAK3, and TYK2, as well as many STAT proteins, including STAT 1, STAT 2, and STAT3 (Simon et al., 2021). The pathway is started when a ligand or cytokine interacts with a receptor on the cell membrane, acting as an extracellular signal. This results in a structural or conformational change, which then activates the relevant JAK isoforms, which can be homodimers or heterodimers (Malemud, 2018).

The JAK auto-phosphorylation mechanism produces a docking site for the STAT protein, which upon binding also undergoes phosphorylation. The STAT proteins are transported or translocated into the cell nucleus by the JAKs, which triggers the start of gene expression and protein synthesis. Janus Kinase inhibitors JAKS1, JAK2, and TYK2 have strong interaction with IL-1B and produce acute phase inflammatory response (Harrington et al., 2020).

IL-1β is a cytokine with pleiotropic in structure that have a significant role in the pathophysiology of inflammatory development of arthritis, such as the highly prevalent disorder of rheumatoid arthritis. (Dinarello, 1996). In order to boost the production of endothelial adhesion molecules, cause inflammation, and activate stromal and hematopoietic cells, two members of the IL-1 subfamily named as IL-1α and IL-1β, stimulate several pathways (Levescot et al., 2021).

1.1.5 Diagnosis of RA

A thorough likelihood-based physical examination and patient history are necessary for the diagnosis of RA. According to a study, the American College of Rheumatology (ACR) and the European League Against Rheumatism (EULAR) jointly presented the diagnosis criteria of RA in 2010 (Browning, n.d.)

With no other diagnosis that more fully explains the synovitis, these suggestions are meant to diagnose RA in individuals who have synovitis in at least one joint. The quantity and location of the afflicted joints, as well as serologic abnormalities (the presence of anti-citrullinated peptide), are all factors for the rise of inflammatory markers, and the length of symptom persistence (Littlejohn & Monrad, 2018).

Pain, swelling of the joints and stiffness, the presence of RF, as well as possible bone and cartilage deterioration, are the main signs and symptoms of RA (Kung & Bykerk, 2014) In addition to the joints, other organs such as the blood vessels, kidneys, heart, lungs, and liver may also be impacted (Jutley et al., 2017). RA can also result in malaise, exhaustion, and weight loss. Several antibodies like anti-citrullinated protein (ACPA), rheumatoid factor (RF) are used as biomarkers for diagnosis of RA (van Delft & Huizinga, 2020).

1.1.5.1 Clinical Testing: When RA is clinically suspected, laboratory testing must be requested to help with knowledge of diagnosis and gauge the severity of the condition (Scott, 1992). Some of the tests covered in this list are rheumatoid factor (RF), antibodies to cyclic citrullinated peptides (anti-CCP Abs), including some inflammatory markers like erythrocyte sedimentation rate (ESR) and CRP, and basic laboratory work like complete blood count and complete metabolic panel (Aletaha & Smolen, 2002). The RF and anti-CCP antibodies tell a patient as if they are having "seropositive RA". In combined meta-analyses, the more modern anti-CCP test has a sensitivity of approximately 67% and a specificity of 97% for RA (Littlejohn & Monrad, 2018).

1.1.5.2 Anti-MCV antibody as a diagnostic marker: Antibodies for mutant citrullinated vimentin (MCV), in addition to RF and ACPA, may be helpful supplementary biomarkers in a variety of diagnostic tools for RA (T. Zhu & Feng, 2013).

In the synovium of RA patients, a protein produced from apoptotic macrophages is present that is recognized by anti-MCV antibodies. Anti-MCV antibodies can serve as a reliable diagnostic marker for RA, and according to a meta-analysis, they have diagnostic values comparable to that of anti-CCP and RF (Jang et al., 2022). Joint destruction is hardly evident in the very early stages of the disease, despite structural abnormalities, which can be seen by standard radiography or other imaging techniques, being the best way to distinguish RA from other rheumatic conditions (Aletaha et al., 2010).

1.1.5.3 Diagnostic tools: Ultrasonography is used as a diagnostic tool for early rheumatoid arthritis. Besides this, Computed Tomography (CT)and Magnetic Resonance Imaging (MRI) and are some of the tools for the diagnosis of RA (Boylan, 2020).

To identify soft tissue inflammation and synovitis (particularly tenosynovitis) before the onset of joint injury, plain radiographs are less sensitive than ultrasonography and magnetic resonance

imaging (MRI). To help t in the early diagnosis and cure of RA patients, rheumatologists are increasingly receiving training in point-of-care musculoskeletal ultrasonography (Sparks, 2019).

1.1.5.4 DAS-28 score Test.

Disease activity score DAS-28 is a type of scoring method used to identify treatment effectiveness and evaluate the disease activity of RA patients accounts in daily life. There are 4 parameters needed, 2 of which are subjective criteria like sore joints (range 0-28) and 2 of which are main factors like swollen joints (range 0-28) (Jensen Hansen et al., 2017).

Fig 1.1.6: Diagnosis of RA using DAS-28 calculator showing high disease activity (https://www.cliexa.com/2016/02/das28-patient-physician-laboratory-collaboration-optimizing-patients-outcomes-using-cliexa-ra/).

Table 1.1: Criteria for classification and diagnosis of Rheumatoid Arthritis demonstrates that 4 out of the 7 found in RA patients (Arnett et al., n.d.).

Sr#	Symptoms	Diagnostic Criteria of Rheumatoid Arthritis
1	Morning stiffness	Morning stiffness in joints that persists >1 hour before it improves to its maximum
2	Arthritis in hand joints	Swelling in one of the wrists or MCP joints
3	Arthritic Joints	Arthritis of 3/14 joints
4	Symmetric arthritis	The identical joint locations on both sides of the body are simultaneously affected
5	Serum rheumatoid factor	Positive RF and positive Anti CCP
6	Rheumatoid nodules	Nodules under the skin, on extensor surfaces, over bony prominences, or in the juxta-articular areas
7	Radiographic changes	Includes bone decalcification or erosion that is localized in or even most pronounced near the affected joint or both.

1.1.6 Treatment of RA

1.1.6.1 DMARDs: With the emergence of biologics and other targeted medicines, as well as more aggressive initial therapy tactics like treat-to-target, the cure of RA has undergone a revolution in past years, with many patients experiencing noticeably better clinical outcomes. Disease-modifying antirheumatic drugs (DMARD) therapy is the cornerstone of the management of RA (de La Forest Divonne et al., 2017).

These can be divided into biologic (b) DMARDs, conventional synthetic (cs) DMARD targeted synthetic (ts) DMARDs, most recently Janus Kinase (JAK) inhibitors (de Cock & Hyrich, 2018; Keating, 2013).

1.1.6.2 NSAIDs: NSAIDs (naproxen, ibuprofen, and coxibs) are used to decrease pain by lowering inflammation during the acute phases. (Rao & Knaus, 2008). Inhibiting cyclooxygenase (COX), particularly COX-2, which is increased during inflammation, is how NSAIDs exert their therapeutic effects. (Hochberg, 2002).

1.1.6.3 Glucocorticoids: Although NSAIDs have a far better safety profile, glucocorticoids (prednisone, hydrocortisone, prednisolone, and dexamethasone) are considered as more effective than NSAIDs due to the complex mechanisms behind their anti-inflammatory and immunosuppressive activities. DMARDs are drugs that prevent or delay joint degeneration and decrease autoimmune activity to encourage remission (del Grossi Moura et al., 2018). Methotrexate (MTX) is listed as a first-line treatment for RA in the 2021 ACR guideline, both as a monotherapy and in combination with other agents, due to its effectiveness, safety record, flexible administration, and affordable price (Radu & Bungau, 2021).

1.1.6.4 bDMARDs: The gold standard of treatment for RA has traditionally been conventional DMARDs (cDMARDs). Methotrexate, either alone or in combination with other cDMARDs, is typically used as first-line therapy. Patients who don't respond good to methotrexate or other cDMARD techniques are advised to add a biological DMARD (bDMARD) (Blair & Deeks, 2017).

Another class of biologic DMARDs utilized for RA treatment is corticosteroids. By binding to the glucocorticoid receptor (GR), also called as nuclear receptor subfamily 3 group C member 1 (NR3C1), corticosteroids reduce inflammation by causing the transcription of several genes that block various inflammatory pathways (Barbulescu et al., 2022).

In addition, natural compounds can change the ratios of transcription factors, such as STAT3, IRF-4 (interferon regulatory factor 4), and the Th17/Treg balance, Foxp3, and important cytokines like IL-1B, IL-6, and TGF (Dudics et al., 2018).

1.1.6.5 IL-1β blocker: The first biological drug created for preventing IL-1β cell signaling was anakinra, a recombinant interleukin 1 receptor antagonist (Y. H. Lee et al., 2009). A

human monoclonal antibody called canakinumab binds to IL-1β exclusively, without interfering with other members of the IL-1 family. There are another interleukin 1 family members outside IL-1β inhibitors, such as Tocilizumab, a recombinant humanized monoclonal antibody aimed against soluble or membrane-bounded IL-6 receptors (Mueller et al., 2021).

Table 1.2: Some biological agents and their mode of action and administration for the treatment of RA. IL-6 interleukin, intravenous Janus kinase (JAK), twice daily (BD), oral (PO), and subcutaneous injection (SC) antitumor necrosis factor inhibition (ANTI-TNF) (Jones et al., 2017).

Agents	Mode of action	Mode of administration
Tofacitinib	JAK inhibitor	PO BD
Infliximab	Anti-TNF	IV 8 Weekly
Certolizumab	Anti-TNF	SC 2 or 4 weekly
Etanercept	Anti-TNF	SC weekly
Adalimumab	Anti-TNF	SC fortnightly

If a patient doesn't respond to csDMARDs, targeted synthetic (ts) or biological DMARDs (bDMARDs) should be added. Biological anti-rheumatic medications include adalimumab, infliximab, certolizumab, sarilumab, and secukinumab. The tumor necrosis factor (TNF-a), interleukin (IL)-6, IL-1β, and IL-17 are only a few of the specific molecules that these monoclonal antibodies target. Additionally, tsDMARDs have targets. For instance, Janus kinases (JAK) are a specific target for the drugs tofacitinib, baricitinib, filgotinib, upadacitinib, and decernotinib (Huang et al., 2021).

1.1.6.6 Non-pharmacological treatments:

Many exercise modalities, psychological interventions, physiotherapy, nutritional interventions, education, and other interventions are included in the various non-pharmacological therapies (NPT). In RA patients, where pharmaceutical therapy options are frequently limited, their complicated action can have a synergistic, additive effect with specific pharmacological interventions (Majnik et al., 2022).

Non-inflammatory symptoms, mostly related to functional impairment, pain, and fatigue, have been demonstrated to be improved by exercise, psychological activities, educational performances, and self-management therapies. Education has also been shown to improve for goal setting and self-management programs (Roodenrijs et al., 2021).

According to studies, RA patients who follow a vegan diet show better improvement, decreased immunoreactivity, lower levels of risky low-density lipoprotein in the blood, and an increase in atheroprotective natural antibodies. Fruits, particularly those high in polyphenols, have been demonstrated to be healthy due to their anti-inflammatory and antioxidant characteristics (Dey et al., 2020).

1.1.7 Risk Factors associated with Rheumatoid Arthritis

1.1.7.1 Genetic and epigenetic risk factors

A population's susceptibility for rheumatoid arthritis can be accounted for in about 60% of instances, per a study's heritability assessments. Genetic marker research showed that the presence of a shared epitope on small regions of the HLA-DRB 0401 and 0404 alleles is linked to the development of rheumatoid arthritis (Lee & Weinblatt, 2001, van Delft & Huizinga, 2020).

Numerous single nucleotide polymorphisms (SNPs) other than those in the HLA region are linked to rheumatoid arthritis. The PTPN22 gene, the second-most significant genetic risk factor for the development of RA, is located at one of these locations (Derksen et al., 2017).

Gene factors may have an impact on how an antigen is processed and presented, how lymphocytes proliferate and differentiate, and how T- and B-cell receptors are encoded. Specific autoantibodies continue to develop depending heavily on HLA haplotypes

(Conigliaro et al., 2016). In RA patients, the elevated peptide-MHC affinity activates CD4+ T cells, which may trigger an autoimmune response to citrullinated self-antigens (James et al., 2010).

1.1.7.2 Non-HLA genes: PTPN22, which codes for the enzyme tyrosine phosphatase, carries the non-HLA genetic variation with the highest risk of developing RA. To prevent protein citrullination, PTPN22 interacts with peptidyl arginine deiminase 4 (PAD4) irrespective of its phosphatase activity (Evangelatos et al., 2019).

Arginine is converted to tryptophan by a cytosine to thymine SNP at position 1858, which also prevents PTPN22 from interacting with PAD4 and causes hyper citrullination in peripheral blood mononuclear cells (Petrovská et al., 2021). Some microRNA gene variations are linked to an increased risk of RA (Filková et al., 2014).

Interleukin-1 (IL-1) also plays a significant role in the destruction of cartilage and synovial inflammation in RA. Unresponsive to signals, the naturally occurring anti-inflammatory protein antagonist (IL-1Ra) can bind to IL-1 receptors. IL-1Ra is essential in mice models of arthritis (Carreira et al., 2005).

1.1.7.3 Environmental factors

A study reported that RA can develop or worsen because of smoking. Smoking has been linked to RF- or ACPA-positive RA (Alaya et al., 2018). Patients with RA who have developed silicosis, also known as Caplan's syndrome, are at risk for developing the rare illness rheumatoid pneumoconiosis, which is brought on by protracted exposure to silica (Croia et al., 2019). Dietary factors affect RA, and research has indicated that vegetarian diets and fasting intervals can slow the progression of RA (Radu & Bungau, 2021; Jin et al., 2021).

Hormonal exposure, tobacco usage, microbiological exposure, smoking, and consuming more than three cups of decaffeinated coffee each day are some of the environmental influences. Tobacco usage provides the strongest evidence for a link between these (Birch & Bhattacharya, 2010). The two main environmental risk factors for the formation of ACPA in RA patients are tobacco use and local microbial pathogenicity (Gómez-Bañuelos et al., 2019).

1.1.7.4 Role of hormones

Low amounts of adrenal and gonadal androgens, however less amount or levels of the immune-suppressive androgens may be pathogenic, as evidenced by the discovery of dihydrotestosterone (DHT) and dehydroepiandrosterone (DHEA) in fluids such as blood, synovial fluid, and in smears of male/female Rheumatoid arthritis (RA) patients (Cutolo et al., 2002).

Aims and Objectives:

Objectives of the study are

- o Evaluation of the susceptibility of IL-1β gene polymorphisms in RA patients
- o To estimate the progression of disease in Pakistani RA patients

CHAPTER #2   REVIEW OF LITERATURE

Review of Literature

Dihydrotestosterone (DHT) and dehydroepiandrosterone (DHEA) were found in fluids like in blood, synovial fluid, and smears of male and female Rheumatoid arthritis (RA) patients, suggesting that decreased levels of the immune-suppressive androgens may be pathogenic (Radu & Bungau, 2021).

In patients with RA, pain and swelling occurring in the hands and feet are common complaints. The swelling is particularly noticeable in the wrists, metacarpophalangeal, metatarsophalangeal, and proximal interphalangeal joints. Additionally, morning stiffness that lasts for several hours, generally longer than 30 minutes (Crowson et al., 2018)

RA influences a person's quality of life, productivity, and physical performance. 40% of patients who receive insufficient care will be unable to work after the disease's first 10 years, and 80% of patients will have misaligned joints (Aletaha & Smolen, 2018).

Up to 1-3% of people have RA, and as people get older, the 3:1 female preponderance gradually disappears. The synovial-lined joints are gradually and irreversibly damaged in RA, leading to deformity and loss of joint space, bone, and function (Turkcapar et al., 2006). Asymmetric arthritis is a hallmark of RA. Joint swelling and palpable discomfort are examples of articular and periarticular symptoms, which also include morning stiffness and substantial mobility limitation in the affected joints (Grassi et al., 1998).

The frequency and incidence of RA differ across ethnic groups in some geographical areas. For instance, the frequency of RA is low in some rural areas of Africa and high among Pima Indians. Geographical location affects the incidence and prevalence of RA within ethnic groups (Tobón et al., 2010b).

2.1 Role of cytokines

Strong hereditary factors have a role in RA. For instance, twin studies have suggested that the heritability of RA is 60%. Only 12–15% of diseases are shared by identical twins, indicating that non-coding parts play a significant role in susceptibility (Smolen et al., 2018).

T lymphocytes, B cells, and macrophages commonly enter the synovial membrane, causing fibroblast-like synoviocytes (FLS) to over-proliferate and obliterate bone and cartilage as a

maladaptive response to injury. Patients with RA have a subset of autoantibodies that are frequently reactive and directed for post-translational modifications of proteins. A confluence of genetic, epigenetic, and environmental factors makes a person susceptible to autoimmunity and the emergence of inflammation in joints (Qiu et al., 2021).

Rheumatoid arthritis treatment has advanced in such a way during the past two decades that it has become a true model of medical achievement. While initial treatment with csDMARDs alone was preferable to an anti-TNF plus MTX. MTX with glucocorticoids influenced the progression of joint deterioration that was equal to that of an anti-TNF plus MTX (Smolen, 2020).

Methotrexate, hydroxychloroquine, leflunomide, chloroquine, and gold salts are a few examples of conventional synthetic (cs) DMARDs that should be used as soon as RA is identified as the first course of treatment. Significantly, patients prefer to take methotrexate. The popularity of csDMARDs is a result of their affordable pricing and effective use (Huang et al., 2021).

IL-1β blocker like Anakinra is the best treatment because the IL-1 receptor is present in almost all tissues and the antagonist blocks the interaction of either IL-1α or IL-1β (Allantaz et al., 2007). Anakinra must be administered daily via injection because of its 6-hour half-life. Similarly, the blockade of TNFα plays an important role in the treatment of RA within minutes of an intravenous infusion. By lowering disease-related pain, weariness, and sadness, this feature most likely accelerates the effectiveness of clinical outcomes (Dinarello & van der Meer, 2013; Maini et al., 1995; Turkstra et al., 2011).

2.2 Factors for initiating Autoimmunity in RA

DNA methylation is a commonly studied epigenetic factor that has a significant part in the pathophysiology of RA. Most past epigenome-wide association studies have solely focused on methylation level, lacking the integration of DNA methylation with mRNA expression needed to fully find the pathophysiological factors and the functional activities of DNA methylation in RA (H. Zhu et al., 2019). DNA methylation, which occurs when DNA methyltransferases (DNMTs) add a CH3 group to the cytosine 5 site, is the most researched epigenetic change (Hashimoto et al., 2021).

Rheumatoid arthritis is a chronic illness marked by polyarthritis, autonomous synovial growth leads to osseocartilaginous destruction (Tanaka et al., 1998). Studies have shown that proteinases, some cytokines, and several auto-antibodies contribute to the development of this disease even though the pathophysiology of RA is still unknown (Noack & Miossec, 2017).

It has been observed that the disease-affected joints overexpress proteinases like calpains, cathepsins, and matrix metalloproteinases (MMPs), which are thought to be a main factor in the osseocartilaginous destruction. Moreover, cytokines are now widely play significant roles in RA, as an increasing number of studies have shown (Yoshihara et al., 2000).

Trials for treatment of RA with biological treatments against tumor necrosis factor-a (TNF-), IL-1β, and IL-6 reportedly had favorable outcomes. Finally, it has been demonstrated that several autoantibodies, including those against follistatin-related protein (FRP), glucose-6-phosphate isomerase, and citrullinated proteins, are involved in the pathogenesis of RA (Yoshifuji et al., 2005).

2.3 Glucose metabolism in RA

Recent data shows that the etiology of RA includes glucose metabolism as a critical factor. Glycolytic breakdown occurs in inflammatory effector cells. Macrophages from people with RA exhibit high mitochondrial activity and are good at producing ROS (Garcia-Carbonell et al., 2016).

Suppression of GSK-3b increases ATP synthesis, ROS production, and mitochondrial activity in macrophages taken from patients. This metabolic arrangement results in the translocation of the glycolytic enzyme pyruvate kinase 2 (PKM2) from the cytoplasm to the nucleus. One of the functional effects is the PKM2-dependent activation of STAT3, which boosts the activation and release of pro-inflammatory cytokines like IL-6 and IL-1. Such inflammatory macrophages coexist with T cells in the joint, where they compete for need of glucose availability (Qiu et al., 2021).
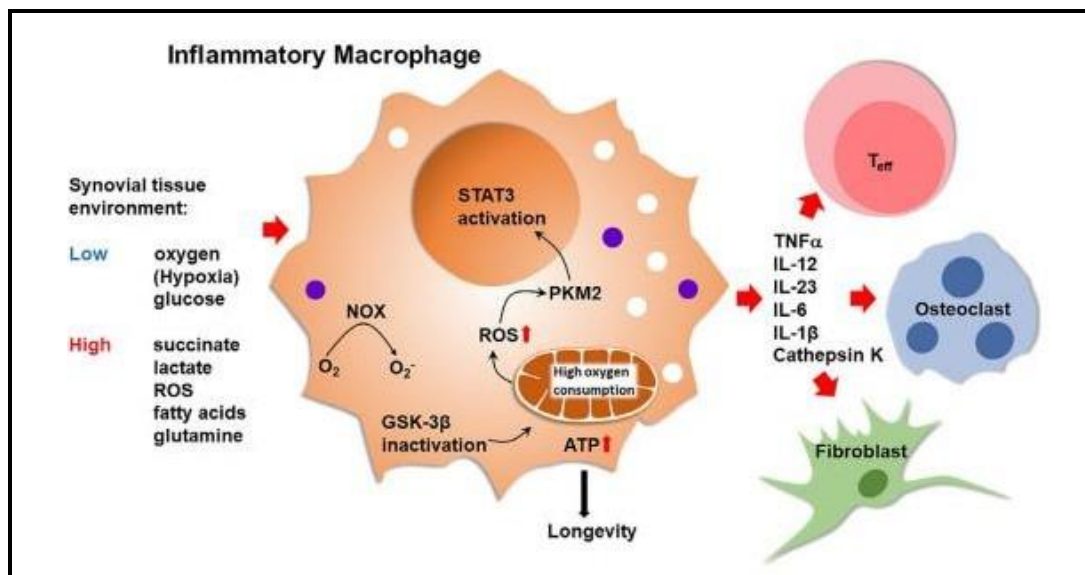
Fig 2.1: Rheumatoid synovitis inflammatory macrophages. TNFα, IL-23, IL-6, and IL-1β produced in response to high mitochondrial activity. Through altering the actions of nearby T effector cells, endothelial cells, osteoclasts, and synovial fibroblasts, inflammatory macrophages cause and maintain synovitis. NADPH oxidase (NOX), T effector cell (Teff).

2.4 Role of autophagy in RA

Upregulated autophagy causes Fibroblast-like synovitis (FLS) to become activated, grow, and proliferate, which in turn encourages RA-associated synovitis. The blocking of excessive immune cell activation and cytokine generation by FLS apoptosis is noteworthy since it functions as a core mechanism for reducing inflammation (Zhao et al., 2021). It has been found that RA patients' synovia exhibits a marked reduction in FLS apoptotic rate (Karami et al., 2020).

Synovial hyperplasia, FLS infiltration of cartilage, and subchondral bone deterioration are some symptoms of RA. These findings show that FLS' resistance to apoptosis, which is normally mediated due to autophagic processes, is the cause of the growing loss of bone and cartilage (Celia et al., 2022).

It was evaluated that autophagy- and apoptosis-related indicators in patients with RA and osteoarthritis and found that RA patients mostly experience synovial lining hyperplasia. In

contrast to OA patients, RA patients' synovial linings exhibit upregulated or high expression of Microtubule-associated protein light chain 3 (LC3). Moreover, RA patients had significantly more apoptotic cells in their synovial lining than OA patients did. An important part of immune activities, self-tolerance, can be encouraged by autophagy (Feng et al., 2016).

2.5 Bone erosion in RA

Cells of the osteoblastic family (osteocytes, lining cells, and maybe other cells) control bone turnover in addition to their capacity to generate matrices. They respond to growth factors, prostaglandins, insulin, parathyroid hormone, glucocorticoids, vitamin D, sex steroids, and other substances. There are numerous cytokines that are produced locally and may regulate bone resorption like Prostaglandins, IL-lβ, TNFα. Moreover, osteoblastic cells create and react to a variety of growth for various growth hormones on, remodeling, and repair. The skeletal reaction to mechanical force is likely also mediated by cells of the osteoblastic lineage (Rodan, 1992).

A highly rich source of myeloid precursors, immunomodulatory, pro-inflammatory, and osteoclastogenic factors is the pannus and inflammatory synovium. RA synovium-produced substances, particularly RANKL and macrophage colony-stimulating factor (M-CSF), can lead to macrophage lineage cells to differentiate into osteoclasts. The process of bone remodeling is shown in Fig 1.3 (Favero et al., 2014).
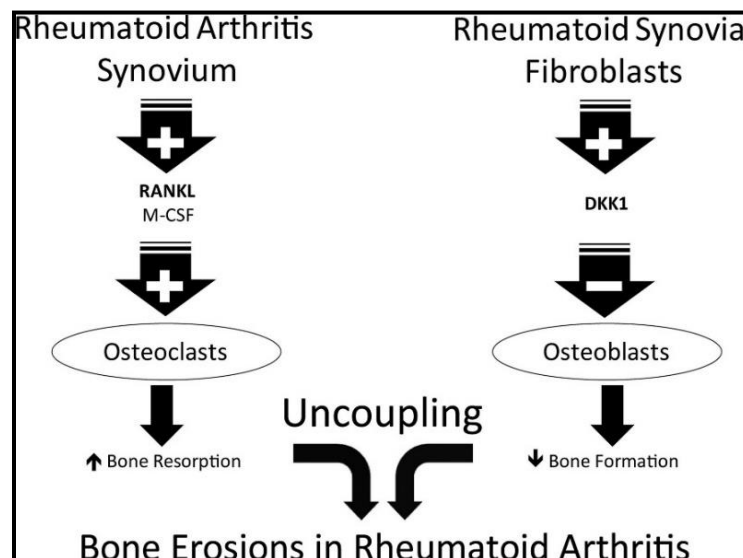
Fig 2.2: The schematic diagram of bone remodeling and bone erosion in RA. Bone erosion and bone remodeling are not linked to rheumatoid arthritis. Bone erosion develops in rheumatoid arthritis patients due to high bone destruction and a decrease in bone growth consequently leading to the formation of bone erosion.

Physiological bone remodeling requires a balance between bone-forming osteoblasts cells and bone-resorbing osteoclasts. RANKL (receptor activator of nuclear factor kB ligand) activates osteoclast precursor cells too much of it causes accelerated bone resorption (Rana et al., 2018).

The issue with RA bone is caused by the release of RANKL by TNF-stimulated synovial cells, which puts osteoclasts and hence bone resorption into overdrive. Pharmacological RANKL inhibition can halt the advancement of RA bone erosions but does not cure bone erosions because osteoblast bone formation is still restricted in RA joints as shown in fig (Rauch & Adachi, 2016).
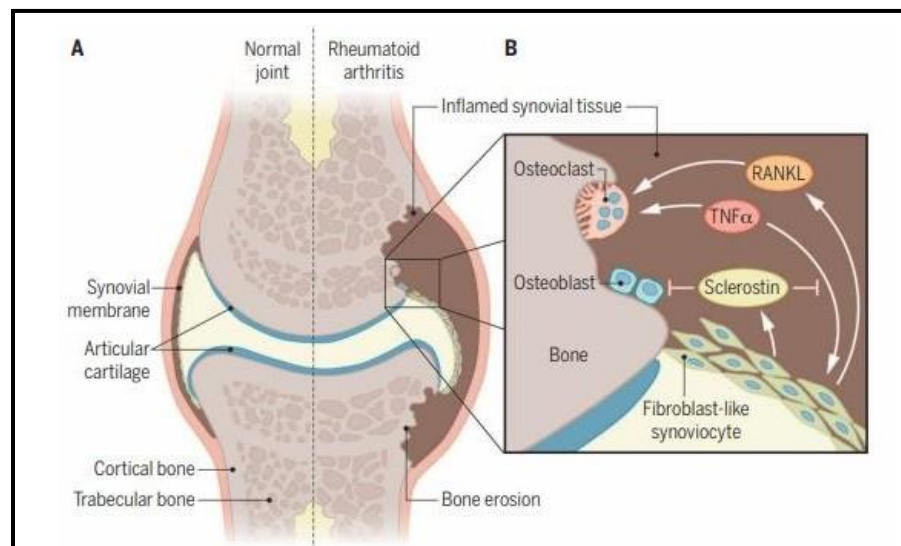
Fig 2.3: Sclerostin action in RA-related bone erosions. One of the crucial molecules in the inflammatory process is TNF, which is produced by a range of cell types in the inflamed synovium. TNF causes osteoclasts to break down bone by themselves, and fibroblast-like synoviocytes release more RANKL, both of which encourage bone resorption. TNFα also causes these synoviocytes to secrete sclerostin, which suppresses TNFα signaling in

synoviocytes and inhibits osteoblastic bone growth. Sclerostin thus indirectly aids in limiting osteoclast activity's capacity to resorb bone.

2.6 IL-1 Family

The precursor proteins for IL-1α and IL-1β, pro-IL-1α and pro-IL-1β, are 31 kDa proteins that are later degraded by cellular proteases into the mature 17 kDa protein. It is believed that IL-1α functions as an autocrine messenger even though it is normally maintained inside of cells or expressed on the surface of cell (Dinarello, 2019).

On the other hand, IL-1β is released and has an impact on other cells in a biological way. The 17 kDa protein IL-1Ra is produced and secreted by the same types of cells that express IL-1β. It is believed to be essential for reducing the effects of IL-1β in both chronic and infectious disorders because of cytokines, viral products, and other acute-phase proteins all increase the manufacture of IL-1Ra (Migliorini et al., 2020).

Target cells' surface receptors are highly affinely bound by every member of the IL-1 family. Cellular responses are a result of IL-1α or IL-1β binding to type I IL-1 receptors (IL-1RI), which is enhanced by the accessory protein IL-1R-AcP. This binding to IL-1RI leads to the control of gene expression and intracellular signal transmission. interaction to type II IL-1 receptors (IL-1RII) on these cells does not result in cell activation because these receptors have a relatively short cytoplasmic region that is unable to convey signals following IL-1β interaction. As a result, IL-1RII serves as a bogus receptor. Although IL-1Ra binds to IL-1RI and IL1RII, contrary to what its name implies, it does not cause signal transduction or activation as shown in **Fig1.5** (Kay, 2004). Instead, it inhibits IL-1α and IL-1β binding by competitive antagonism, decreasing the effects of these cytokines.
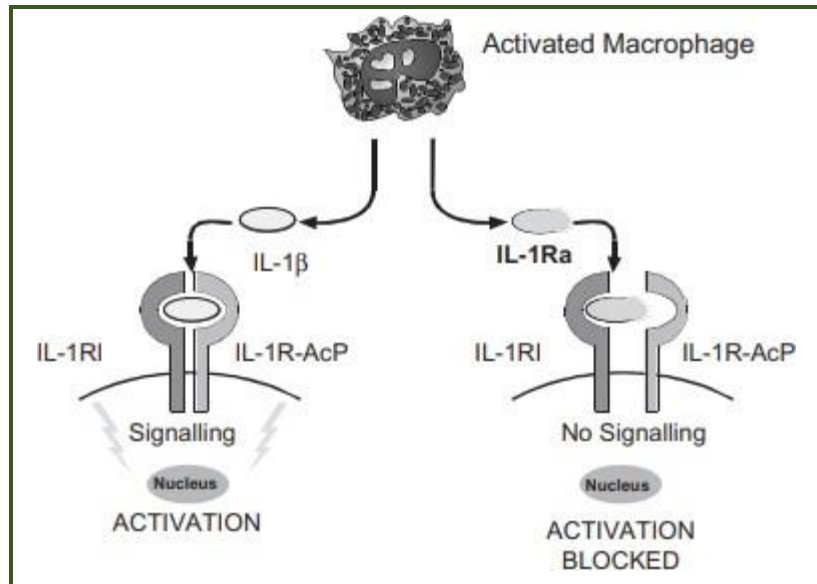
Fig 2.4: Cellular response induced by IL-1β by binding to its receptors. 1 IL-1 receptor causes cellular activation whereas IL-1Ra blocks these IL-1β induced responses by binding IL-1RII because it has a short cytoplasmic domain.

2.7 Role of Interleukin 1-Beta (IL-1β) in RA

Blocking the expression of IL-1β is thought to permanently lessen the severity of the ailment because IL-1β is a known chronic inflammatory mediator in RA. Additionally, the equilibrium between IL-1β and its antagonist (IL-1Ra) may affect how RA manifests itself. The genes that produce IL-1α, IL-1β, and IL-1Ra are situated on the long arm of chromosome 2 (You et al., 2007).

These genes have been found to have several polymorphisms, some of which are functional and impact their secretion and, consequently, their susceptibility to disease. Examples include TNF, IL-1Ra Variable Number Tandem Repeats (VNTR), and IL-1 (511C>T). (Lagha et al., 2015).

The members of the IL-1 family are IL-1α and IL-1β receptor antagonists (IL-1Ra). In RA, IL-1α and IL-1β operate as agonists and are linked to joint degeneration. Contrarily, IL-1Ra has been demonstrated to prevent joint erosions in RA by acting as an antagonist in IL-1β signaling by inhibiting the binding of IL-1β to IL-1 R type I. Numerous high-degree sequence differences exist in the IL1β and IL-1RN genes, and these variations may have a big impact

on RA susceptibility. Intron 2 of the IL-1RN gene has 86 bp-long variable number tandem repeats (VNTR), and IL1RN allele 2 is linked to autoimmune diseases (C.-G. You et al., 2007; Arman et al., n.d.).

The IL-1β gene family consists of three identified genes that are in a 430 kb area on the long arm of human chromosome 2 (2q13). In RA synovial fluid, elevated levels of IL-1α and IL-1β have been detected. It has been demonstrated that circulating levels of IL-1β correspond with disease activity and radiographic progression (Buchs et al., 2001). Exon V (at +3954) and the promoter region (at 511) of the IL-1β gene have polymorphisms that have been linked to IL-1β production. A single base change (C/T) in exon 2 at position +2018 and a VNTR in intron 214 of the IL-1Ra gene are polymorphisms (Allam et al., 2013).

Metalloproteinase genes, including those for collagenases and elastases, adhesion molecules, and several proinflammatory mediators involved in joint degeneration are all induced by IL-1β. In a different RA animal model, antibodies against IL-1β reduce collagen-induced arthritis. RA patients with active disease had greater blood and synovial IL-1β concentrations than those in remission. Moreover, IL-1Ra has been utilized to treat RA patients (Akash et al., 2013) (Camargo et al., 2004).

The synthesis and activity of IL-1α and IL-1β are regulated by a natural competitive antagonist, transcriptional processes, and post-translational mechanisms (Luotola, 2022). IL-1 has been discovered to be strongly expressed at the cartilage-pannus interface, even though IL-1 and IL-1 mRNA have both been recovered from RA synovial fluid. Four IL-1 gene cluster indicators were discovered to be linked to erosive RA in a study of sib-pair RA families, especially in sib-pair families with no chance of sharing two HLA-DRB1 alleles identical by descent (Harrison et al., 2008).
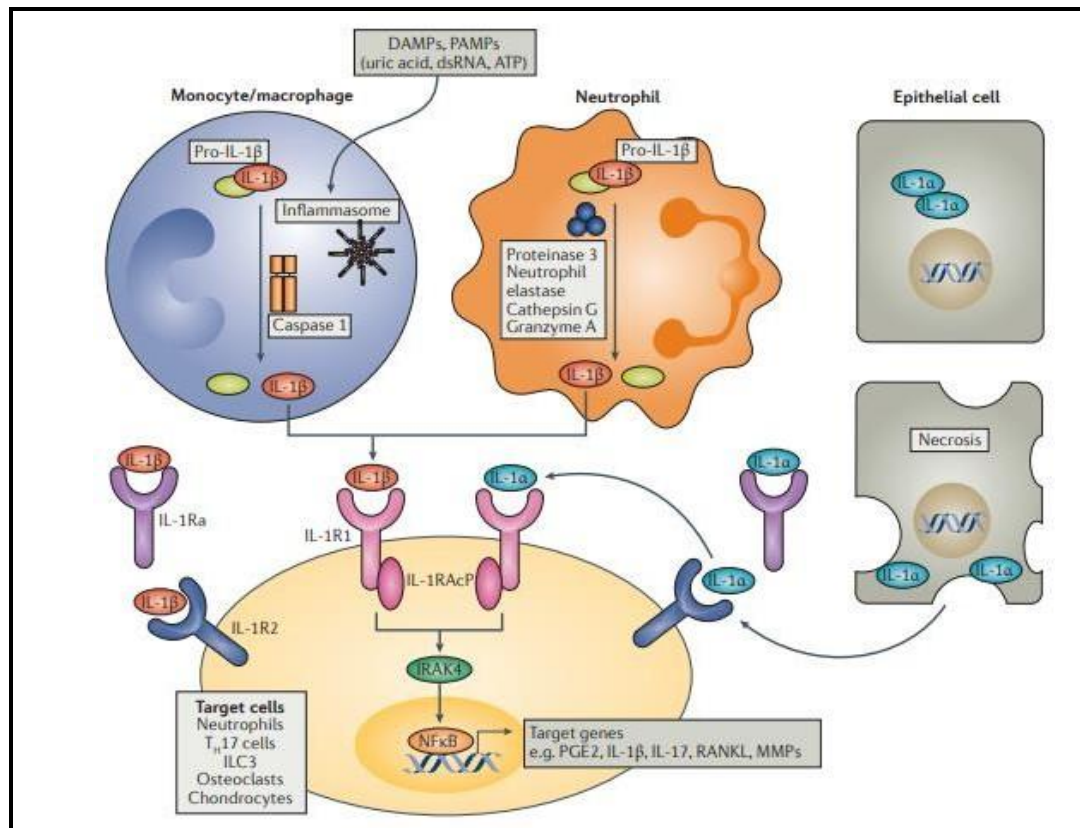
Fig 2.5: 1: IL-1β signaling. Enzymatic processing of pro-IL-1, the intracellular precursor protein, is required for the release of IL-1 from neutrophils, monocytes, and macrophages (Schett et al., 2016).

2.8 SNP characterization and *in-silico* research

DNA structure, a significant part of phenotypic diversity is contributed by polymorphism, which also offers a way for natural selection to cause microevolutionary change and species divergence. To find and characterize DNA polymorphism and divergence among functional and nonfunctional sequences, more recently related genomes have been sequenced (Doniger et al., 2008).

SNPs are genetic variants that only contribute 0.1% of population differences. SNPs are genetic variations that only contribute 0.1% of population differences. Around 50% of the SNPs in the coding area are missense SNPs, while the remaining 50% are silent or synonymous (Akhtar et al., 2021).

Non-coding SNPs can influence the stability of mRNA and the activity of its promoter by generating or destroying miRNA sites, which results in altered gene expression and the up-or down-regulation of a gene (Choi et al., 2012).

These in silico investigations are highly helpful in finding the high-risk SNPs that may harm the structure and function of the gene, leading to the translation of the defective protein that would ultimately be causing diseases, in addition to genetic association studies. These *in silico* research is important because it enables us to reveal hidden characteristics of genes and their variants. With the use of these findings, the functional implications of genes may also be calculated, which will aid in identifying new disease pathogenic pathways and developing novel treatment plans for the illnesses.

IL-1β gene is 14021bp in length. Encoding 8 exons and 269 amino acids. IL-1β gene encoding deleterious SNPs located in promoter regions have great effect on gene regulation. A total of four SNPs located in intergenic and in intronic regions were selected for analysis for susceptibility of these SNPs in Pakistani population.

As IL-1β is crucial to the etiology of RA. The elevated levels of this cytokine found in RA patients highlight the significant function that this cytokine serves. As was previously mentioned, this cytokine controls the amounts of numerous other cytokines. It's possible that genetic variation in this cytokine's regulatory regions will have an impact on how it is expressed. The illness susceptibility and severity of RA may be affected by the changed expression. The relationship between four distinct polymorphisms in the IL1β genes intronic and intergenic region and the Pakistani population was examined. This was done using allele-specific PCR, and the results were statistically examined to see if there was any evidence of a link between the polymorphisms and RA.

CHAPTER #3   METHODOLOGY

Materials and Methods

3.1. Study subjects

The underlying study was conducted to analyze the susceptibility of the IL-1β gene and its four polymorphisms (**rs2853550, rs16944**, **rs1143643**, **rs3136558**) in the population of patients having RA in comparison with healthy controls. A total of 140 samples (cases= 100, controls = 40, controls were 35 year and above) were collected for this study along with thorough history taking and clinical and laboratory data. The sampling was done from the and Al—Shifa diagnostic, Combined Military Hospital (CMH) Rawalpindi, Pakistan Institute of Medical Sciences (PIMS), Islamabad over a period of 2 to 3 months. Clinical diagnoses of the afflicted participants were made by qualified rheumatologists through physical examination and analysis of screening test data to clinically diagnose the affected people Table 3.1. The participants were of Pakistani descent and informed consent was taken before being recruited. The study was done after taking approval by the review board of Pakistan's National University of Sciences and Technology (NUST), Islamabad.

3.1.1 Characteristics of study subjects for SNPs: Percentages of females included in case group for analysis of these SNPs were 80 percent and percentages of males were 20 per cent. The mean age of individuals included in the study was 40.4±9.3 years. The female's percentage in the control group was 77 per cent. The percentage of males was 23 percent and average of individual included in study was 31.5±10.75.

Table 3.1: Characteristics of sample patients involved in study

SNP ID No.	Age (Years)	Gender		Age of onset			Duration of symptoms			Disease severity	
		Male (%)	Female (%)	<30 yrs (%)	30-40 yrs (%)	>40 yrs (%)	>6 weeks	>6 months (%)	>1 year (%)	Moderate (%)	Severe (%)
• rs2853550 • rs16944 • rs1143643	40.4±10.3	20	80	20	45	35	4	16	80	35	65

• rs3136558												

3.2 Blood sample collection

The blood was collected from RA patients in appropriately labeled EDTA vials of 5ml with name, age, and identification numbers. Before the blood was extracted, the informed consent of patients was obtained. During blood sampling, the necessary mentioned criteria were appropriately evaluated. The samples were returned to the lab after being promptly put in an ice box and the samples were kept at 4°C.

Table 3.2. List of materials required for blood sampling

Sr #	Material for blood sampling
1	5ml EDTA Vials
2	Tourniquet
3	Syringes
4	Adhesive Bandages
5	Ice Box
6	Alcohol Pads
7	Gloves

3.3 Genomic DNA extraction

Blood samples were taken to ASAB Immunogenetics lab, NUST, Islamabad, and isolation of genomic DNA from blood samples was performed by using the Phenol-Chloroform Protocol of two days. All the materials and glass wares used for extracting DNA were washed, cleaned, and autoclaved at 120°C and 15psi pressure. The following are the tables for materials and recipes for the extraction of genomic DNA.

3.3.1 Reagents required for DNA extraction

Table 3.3. Solution A (blood lysis)

Sr #	Components	Molarity (mM)	Quantity (g/1000ml)
1	Tris	10	1.20
2	Sucrose	0.32	109.44
3	$MgCl_2$	5	1.0
4	Distilled Water	-	1000

Solution A was autoclaved but before using solution A, Triton 100X (1%V/V) was added.

Table 3.4. Solution B (DNA and Protein precipitation)

Sr#	Components	Molarity (mM)	Quantity (g/1000ml)
1	NaCl	400	23.37
2	EDTA	2	0.58
3	Tris	10	1.21

Table 3.5. Solution C (For DNA Isolation)

Sr#	Components	Quantity (µl)
1	Phenol	400

Table 3.6. Solution D (DNA purification)

Sr#	Components	Quantity (ml/500ml)
1	Isoamyl alcohol	20
2	Chloroform	480

Table 3.7. 20%SDS solution

Sr#	Components	Quantity (g/100ml)
1	SDS	20
2	Distilled Water	Upto100ml

20%SDS was dissolved in distilled water up to 100ml to make 20%SDS solution.

3.4 PROTOCOL

3.4.1 Day 1 of DNA extraction

Blood samples were incubated at almost room temperature and mixed well by inverting the tube for several times and then 500µl of the blood sample was taken and added into the 2ml centrifuge tube (Axygen, California, USA). 500µl of solution A was added in a centrifuge

tube which was then closed and inverted 8-10 times and then incubated at room temperature for 10 minutes. In a microcentrifuge, the mixture was centrifuged for 10 minutes at 13000 rpm. The pellet was re-suspended twice in 500 l of lysing solution A after the supernatant was discarded. The pellet was once more centrifuged for 10 minutes at a speed of 13000 rpm. The particle was then re-suspended in 500 l of solution B for precipitation after the supernatant was discarded. 5 l of proteinase K and 20 l of 20% sodium dodecyl sulphate (SDS) were added, and the mixture was then incubated at 37 °C overnight.

3.4.2. DAY 2 of DNA extraction

Solution C and solution D was made fresh and 500μl was added to the previously incubated sample. After that, these tubes were centrifuged at 13000 rpm for 10mins. In a fresh tube, the aqueous layer was collected. The aqueous layer was then mixed with 500 l of solution D before being centrifuged at 13000 rpm for 10 min. A fresh Eppendorf tube was used to capture the aqueous layer. By adding 55 l of sodium acetate (3M, pH 6) and an equivalent volume of isopropanol, as well as repeatedly inverting the tube, DNA was precipitated out. 10 min. of centrifuging at 13000 rpm. The DNA pellet was mixed with 200 l of 100% ethanol and centrifuged at 13000 rpm for 7 minutes. Then 200 l of 70% ethanol was added, and the mixture was centrifuged for 7 minutes at 13000 rpm. After discarding the ethanol, the DNA pellet was dried for 30 minutes at 37°C before being dissolved in water.

3.5 Agarose gel electrophoresis

Gel electrophoresis was used to separate DNA or protein in the agarose matrix. 1% (w/v) agarose gel was resorted to analyze the quality of DNA. The reagents are listed in the following table.

3.5.1 Reagents

 Table 3.8. 10 X TAE Buffer

Sr#	Components	Quantity (g/1000ml)

1	Acetic acid	55
2	Tris base	108
3	EDTA	7.5
4	Deionized water	Upto 1000

The buffer was diluted to make the volume 1000ml and dissolved by using a magnetic stirrer. For the preparation of 1 X TAE buffer, 50ml TAE buffer was dissolved in 450ml distilled water to make the volume of 500ml.

Table 3.9. 1% agarose gel

Sr#	Components	Quantity (g or µl/1000ml)
1	Agarose	1
2	1X TAE	100ml
3	1X Ethidium Bromide	3

3.5.2. Protocol

1g of agarose powder was weighed appropriately on an electronic balance. It was then dissolved in 100ml of 1X TAE buffer in the microwave for nearly 2 minutes. After letting the solution cool down for a while and the steam evaporated, ethidium bromide was mixed in the gel solution for staining. The gel solution was then added to the gel-casting tray, where it was left to set up at room temperature. After putting the gel in a tank with 1 X TAE buffer, the gel was loaded by adding dye, and genomic dye was evenly distributed in wells of the gel. For

40 minutes, the electrophoresis was run at 60 volts. The gel was then examined in Omnidoc to produce an electrogram of the gel.

3.6 DNA Quantification

The isolated genomic DNA was quantified with the help of ThermoScientific Nanodrop 2000 UV-Vis Spectrophotometer and Nanodrop TM 2000 software at ASAB laboratory, NUST. At first, the Nuclease-free buffer was used for blank and then the sample was loaded to arbitrate the absorbance ratio. The absorbance wavelength of 260nm was kept as standard and the optimum absorption of nucleic acid was recorded at this wavelength. The absorbance ratio of 260/280nm was suggestive of the purity of DNA.

3.7   SNPs

Our findings focused to find out the susceptibility of these polymorphisms in Pakistani RA patients. The list of SNPs is in the following table.

Table 3.10 List of SNPs of IL-1β gene

Sr #	SNP ID	Location	Alleles	Consequence
1	rs2853550	2q14	A/G/T	Intergenic variant
2	rs16944	2q14	A/G	Intergenic Variant
3	rs1143643	2q14	C/A/T	Intronic Variant
4	rs3136558	2q14	A/G	Intronic Variant

3.8 Primer Designing

A total of four SNPs were selected for the IL-1β gene that were associated with RA development in different populations. Primers were manually designed for each SNP. Three primers were designed for each SNP (Two Forward primers and one Reverse primer). Two forward primers contained polymorphic nucleotides towards its 3' end and amplification was only done in case of perfect match. One of the possible nucleotides of polymorphism was included in one forward primer, while the other was included in second primer. A common

reverse primer was designed for each SNP. For allele-specific polymerase chain reaction (ASPCR) utilizing tools such as dbSNP, Ensemble genome Browser, Oligocalc, and UCSC genome explorer. FASTA SNP sequence was retrieved by entering SNP ID in dbSNP, a database for identifying gene variations. Hair-pin formation and self-complementarity are verified by entering that SNP sequence in Oligocalc, a software for calculating oligonucleotides. Subsequently, in-silico PCR in reference to the UCSC genome browser is done to verify the specific binding of primers through the target allele, as it calculates the hypothetical effects of PCR and gets to know the amplicon size. Forward and reverse primers are entered to amplify the target DNA sequence, and computational amplification was tested. The following table represents primers for SNPs.

Table 3.11 Allele-specific primer designing. The mutated nucleotides are shown in red color.

Sr#	Database Identification No.	Forward primers (5'-3')	length	Reverse Primers (3`-5`)	Product size
1	rs2853550	CTTCAGCTGATCCTGTTCCAA CTTCAGCTGATCCTGTTCCAG	21 21	TATCCCTTCGCCAACGAGTAGT	412bp
2	rs16944	TGGGTGCTGTTCTCTGCCTCA TGGGTGCTGTTCTCTGCCTCG	21 21	GTCTACTCGAGACGGATTTTAC	240bp
3	rs1143643	TAACTGGGCCCCCAACTTTCC TAACTGGGCCCCCAACTTTCT	21 21	TTCCACAGATAGTTTACTCTC	326bp
4	rs3136558	AGAATCCCGAGCTTCTAAAGA AGAATCCCGAGCTTCTAAAGG	21 21	CGTCTGAGTTCTGATCTTACAC	410bp

3.9 Amplification by using Allele Specific Polymerase Chain Reaction (PCR):

To determine whether there were any polymorphisms in the study population, allele-specific PCR was performed. Two independent PCR reactions were run for each SNP on each sample. One of the two forward primers was present in each reaction mixture, but there was only one common reverse primer. This restricted primer amplification from the primer to the presence of the target nucleotide. The reaction mixture of 20 l for PCR was prepared in 0.2 ml tubes

(Axygen, California, USA) by adding 1 l of sample DNA (50 ng/ l), 2.5 l (10X) of PCR buffer (Fermentas, Burlington, Canada), 1.5 l of 25 mM Magnesium chloride (MgCl2), 1 l of 2 mM deoxyribonucleotide tri For thorough mixing, the reaction mixture was centrifuged at 8000 x g for 30 seconds. Gently tapping removed the air bubbles. The reaction mixture was then subjected to thermocycling conditions using a 2720 thermal cycler (Applied Biosystem).

3.10 Agarose Gel Electrophoresis for analysis of PCR products

Agarose gel electrophoresis was used to analyze extracted DNA and PCR results. A 1% (w/v) agarose gel was created by microwave heating 0.5 g of agarose in 50 ml of 1X TAE buffer for 2 minutes. Ethidium bromide solution (final concentration, 0.5 g/ml) was then added for DNA staining. The gel was allowed to set up at room temperature in a gel casting tray. After that, the gel was put in the electrophoretic device's buffer tank. The extracted DNA was mixed with the loading dye (0.25 percent bromophenol blue in a solution of 40 percent sucrose). The mixture was then meticulously placed into the agarose gel wells. Electrophoresis was performed in 1 X TAE buffer at 100 Volts for roughly 30 minutes.

3.11 Statistical Analysis

Statistical study was performed using GraphPad PRISM 10; San Diego, CA was used to perform the chi-square test (x^2) with the least significant difference test set to be at $p < 0.05$.

CHAPTER #4   RESULTS

Results

IL-1β gene also have a close interaction with its receptors and with other genes that includes CASP-1 IL-16, IL-10. This shows that there is an association of different genes together in pathophysiology of IL-1B inducing RA.
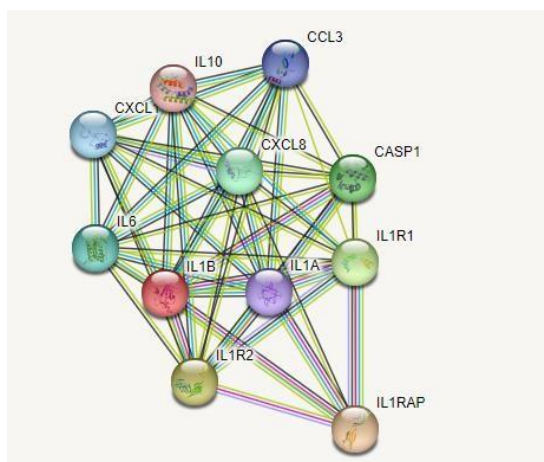
Fig 4.1: IL-1β gene interaction with its receptors and other genes including CASP1 IL-6, IL-10 and other genes obtained through STRING Version 11.5 (https://string-db.org/cgi/network?taskId=bWqHGh2yj6q1&sessionId=biXXcFedM6VU).

Association analysis of four different SNPs located in intronic, and intergenic regions was carried out. RA patients and healthy individuals were screened for the presence of polymorphisms by allele-specific PCR and resulting data was statistically analyzed for any significant association of these polymorphisms with Rheumatoid arthritis.

4.1 Association study of SNP 1 (rs2853550):

This SNP is in the intergenic region of IL-1β gene. By using allele-specific PCR, this polymorphism was examined in 100 patients (cases) and 40 controls (healthy people) (Fig. 4.1). The study patients showed all potential allele combinations (fig. 4.2). Table 4.1 provides an illustration of the genotype and observed allele frequencies. The allele frequencies of people with RA were considerably different from those of healthy controls. Patients and healthy controls exhibit a substantial difference in all genotype frequencies.

Calculations of the Hardy-Weinberg equilibrium (HWE) were done for each group.

When compared to allele frequencies of healthy controls, this shows that there has been a sizable drift in the observed allele frequencies of patients.

4.2 Association study of SNP-2 (rs1143643).

This SNP is in the intronic region of IL-1β gene. By using allele-specific PCR, this polymorphism was examined in 100 patients (cases) and 40 controls (healthy people) (Fig. 4.3). The study patients showed all potential allele combinations (fig. 4.4). Table 4.4 provides an illustration of the genotype and observed allele frequencies. The allele frequencies of people with RA were considerably different from those of healthy controls. Patients and healthy controls exhibit a substantial difference in all genotype frequencies.

For each group, Hardy-Weinberg equilibrium (HWE) calculations were performed.

The case-control study's chi square value is 13.97, and the p-value is 0.0009. When compared to allele frequencies of healthy controls, this shows that there has been a sizable drift in the observed allele frequencies of patients. The genetic variant rs1143643 was found to be strongly associated with RA.

4.3 Association study of SNP-3 (rs16944):

This SNP is in the IL-1β gene's intergenic regions. By using allele-specific PCR, this polymorphism was examined in 100 patients (cases) and 40 controls (healthy people) (Fig. 4.5). The study patients showed all potential allele combinations (fig. 4.6). Table 4.7 provides an illustration of the genotype and observed allele frequencies. The allele frequencies of people with RA were considerably different from those of healthy controls. Patients and healthy controls exhibit a substantial difference in all genotype frequencies.

Calculations for each group's Hardy-Weinberg equilibrium (HWE) were done, and the case-control analysis's acceptable p-value was found to be 0.81. When compared to allele frequencies of healthy individuals, this shows that there has been a considerable drift in the observed allele frequencies of affected individuals.

4.4 Association study of SNP-4(rs3136558)

This SNP is an intronic variant of IL-1β gene. By using allele-specific PCR, this polymorphism was examined in 100 patients (cases) and 40 controls (healthy people) (Fig.

4.9). The study patients showed all potential allele combinations (fig. 4.10). Table 4.10 provides an illustration of the genotype and observed allele frequencies. The allele frequencies of people with RA were considerably different from those of healthy controls. Patients and healthy controls exhibit a substantial difference in all genotype frequencies.

For each group, Hardy-Weinberg equilibrium (HWE) calculations were performed. The case-control analysis's p-value was determined. When compared to allele frequencies of healthy controls, this shows that there has been a sizable drift in the observed allele frequencies of patients. Both groups were also acceptable for additional investigation and research.
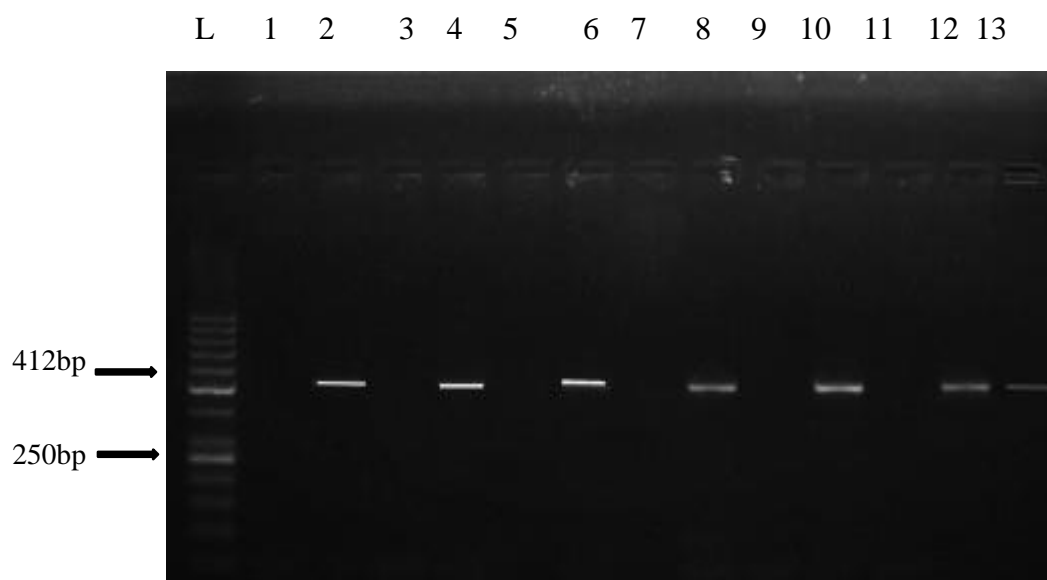
L L: Ladder(50bp).   Lane 1: Negative control   Lane 2: Positive control

Lane 3, 4: Sample 1 (Homozygous for G) Mutant

Lane 5, 6: Sample 2 (Homozygous for G) Mutant

Lane 7, 8: Sample 3 (Homozygous for G) Mutant

Lane 9, 10: Sample 4 (Homozygous for G) Mutant

Lane 11, 12: Sample 5 (Homozygous for G) Mutant

Lane 13, 14: Sample 6 (Heterozygous)

Fig 4.2: Electropherogram of ethidium bromide stained 2% agarose gel for study subjects of SNP-1: The genotype observed in the patient population has been shown for SNP-1. First band of each sample indicates A allele, while second band indicates G allele.

Fig 4.3: Distribution of study subjects across different genotypes for SNP-1: Cases represent RA patients and controls represent healthy individuals. GG genotype of SNP-1 with *p*-value (<0.05) [***], shows a highly significant association of SNP1- with RA in Pakistani patients

Table 4.1: Allele and genotype frequencies calculated for study subjects of SNP-1

Cases	Allele frequencies (%)		Genotype frequencies (%)		
RA patients (n=100)	A	G	AA	AG	GG
	32.28	67.72	17.02	27.43	55.54
Controls (n=40)	65	35	48.34	22.65	30.21

Table 4.2: Hardy-Weinberg equilibrium (HWE) calculations for test population of SNP-1:

	Observed genotype counts			Observed allele counts		χ^2	p-value	Df
	AA	AG	GG	A	G			
RA patients(cases)	16	34	50	32.28%	67.72%	17.75	0.0001	2
Healthy controls	20	10	10	75%	25%			
Total	36	44	60	53.5%	46.5%			

Table 4.3 Statistical association between SNP-1 and Rheumatoid arthritis:

Df	p-value	Association
2	<0.05	Highly significant ***
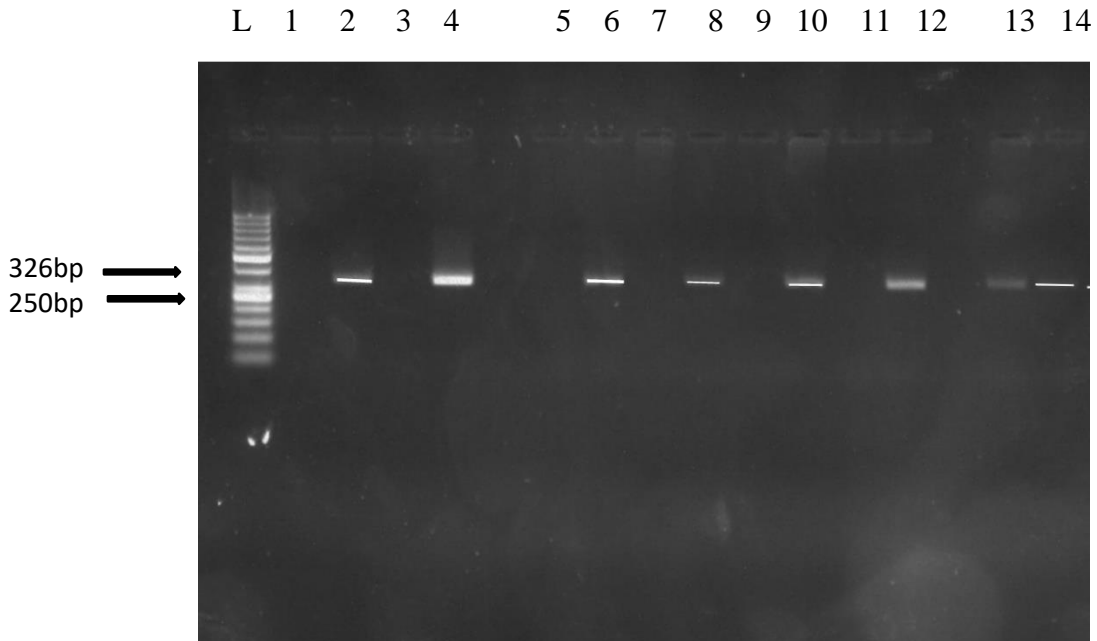
L: Ladder   Lane 1: Negative control   Lane 2: Positive control

Lane 3, 4: Sample 1 (Homozygous for T) Mutant

Lane 5, 6: Sample 2 (Homozygous for T) Mutant

Lane 7, 8: Sample 3 (Homozygous for T) Mutant

Lane 9, 10: Sample 4 (Homozygous for T) Mutant

Lane 11, 12: Sample 5 (Homozygous for T) Mutant

Lane 13, 14: Sample 6 (Heterozygous)

Fig 4.4: Electropherogram of ethidium bromide stained 2% agarose gel for study subjects of SNP-2: The genotype observed in the patient population has been shown for SNP-2. The first band of each sample indicates C allele, while the second band indicates T allele.

Fig 4.5: Distribution of study subjects across different genotypes for SNP-2: Cases represents RA patients and controls represents healthy individuals. TT genotype indicated a significant association of SNP-2 in RA patients with a *p*-value of <0.05.

Table 4.4: Allele and genotype frequencies calculated for study subjects of SNP-2

Cases	Allele frequencies (%)		Genotype frequencies (%)		
RA patients (n=100)	C	T	CC	CT	TT
	39.28	60.72	25.02	33.43	41.55
Controls (n=40)	65	35	40.34	30.65	29.01

Table 4.5: Hardy-Weinberg equilibrium (HWE) calculations for test population of SNP-2:

	Observed genotype counts			Observed allele counts		χ^2	*p*-value	Df
	CC	CT	TT	C	T			
RA patients(cases)	16	34	41	39.28%	60.72%	13.97	0.0009	2
Healthy controls	22	8	10	77%	23%			
Total	36	42	51	58.5%	41.5%			

Table 4.6 Statistical association between SNP-2 and Rheumatoid arthritis:

Df	*p*-value	Association
2	<0.05	Highly significant***
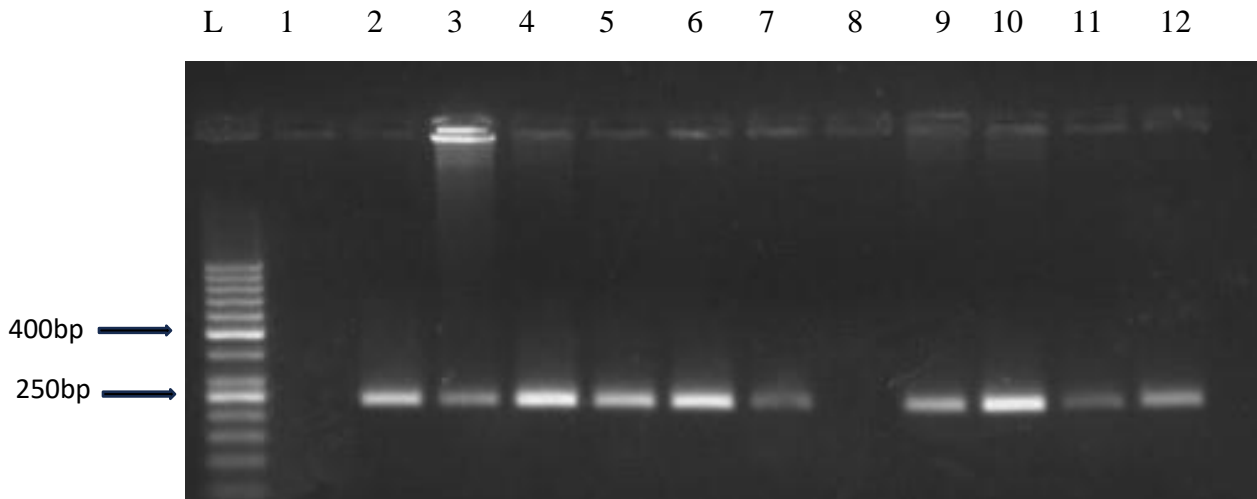
L: Ladder (50bp)   **Lane 1: Negative control**   **Lane 2: positive control**

Lane 3, 4: Sample 1 (Heterozygous)

Lane 5, 6: Sample 2 (Heterozygous)

Lane 7, 8: Sample 3 (Homozygous for A)

Lane 9, 10: Sample 4 (Heterozygous)

Lane 11, 12: Sample 5 (Heterozygous)

Fig 4.6: Electropherogram of ethidium bromide stained 2% agarose gel for study subjects of SNP-3: The genotype observed in the patient population has been shown for SNP-3. The first band of each sample indicates A allele, while the second band indicates G allele.
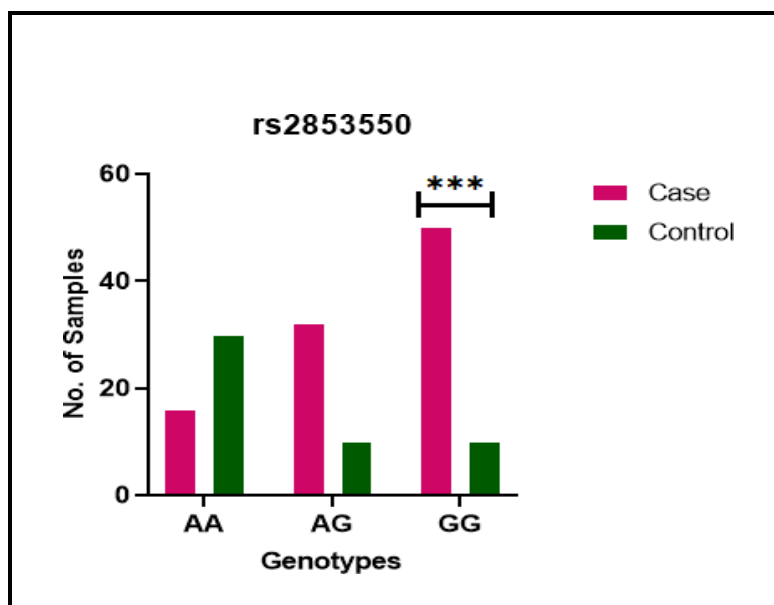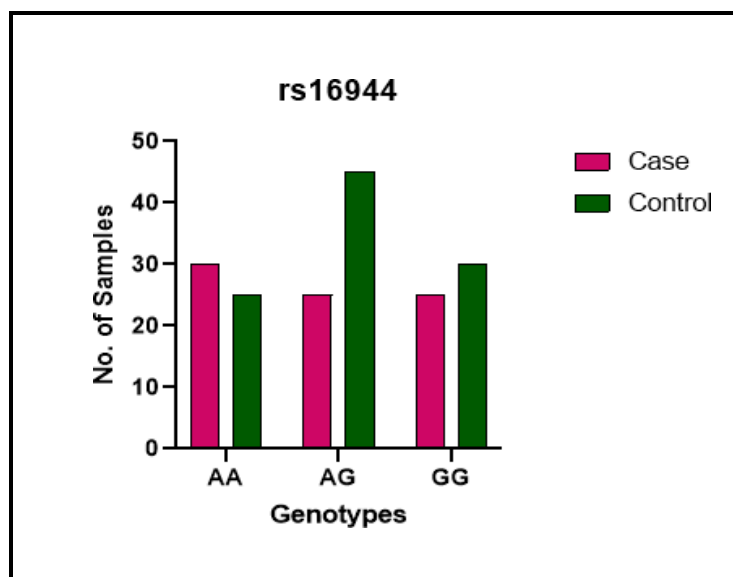
Fig 4.7: Distribution of study subjects across different genotypes for SNP-3: Cases represents RA patients and controls represents healthy individuals. The genotype did not show any significant association of SNP-3 with RA in observed Pakistani population.

**Table 4.7: Allele and genotype frequencies calculated for study subjects of SNP-3**

Cases	Allele frequencies (%)		Genotype frequencies (%)		
RA patients (n=100)	A	G	AA	AG	GG
	70.35	29.65	39.01	40.64	20.35
Controls (n=40)	75.50	24.5	32.86	45.64	21.5

Table 4.8: Hardy-Weinberg equilibrium (HWE) calculations for test population of SNP-3:

	Observed genotype counts			Observed allele counts		χ^2	p-value	Df
	AA	AG	GG	A	G			
RA patients(cases)	35	40	25	70.35	29.65	0.4015	0.81	2
Healthy controls	12	12	6	75.50	24.50			
Total	47	52	31	58.5%	41.5%			

Table 4.9 Statistical association between SNP-3 and Rheumatoid arthritis:

Df	p-value	Association
2	>0.05	Not significant
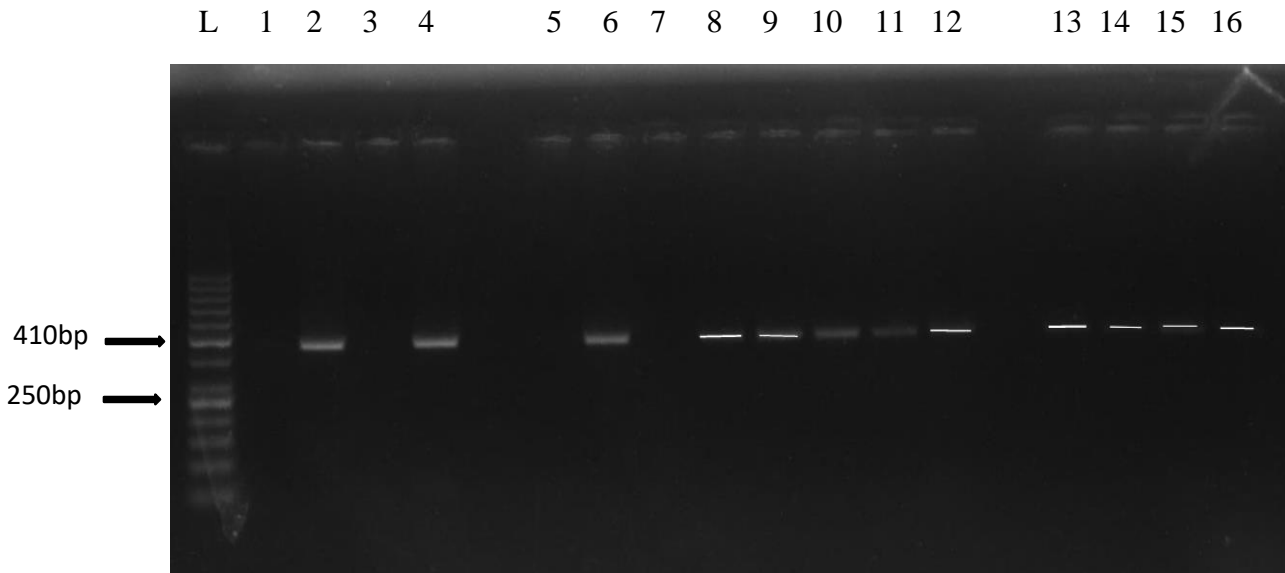
L: ladder   **Lane 1: Negative Control**   **Lane 2: Positive control**

Lane 3, 4: Sample 1 (homozygous for G)

Lane 5, 6: Sample 2 (Homozygous for G)

Lane 7, 8: Sample 3 (Homozygous for G)

Lane 9, 10: Sample 4 (Heterozygous)

Lane 11, 12: Sample 5 (Heterozygous)

Lane 13, 14: Sample 6 (Heterozygous)

Lane 15, 16: Sample 7 (Heterozygous)

Fig 4.8: Electropherogram of ethidium bromide stained 2% agarose gel for study subjects of SNP-4: The genotype observed in the patient population has been shown for SNP-4. The first band of each sample indicates A allele, while the second band indicates G allele. The population of sample has been shifted towards heterozygous genotype for SNP-4.
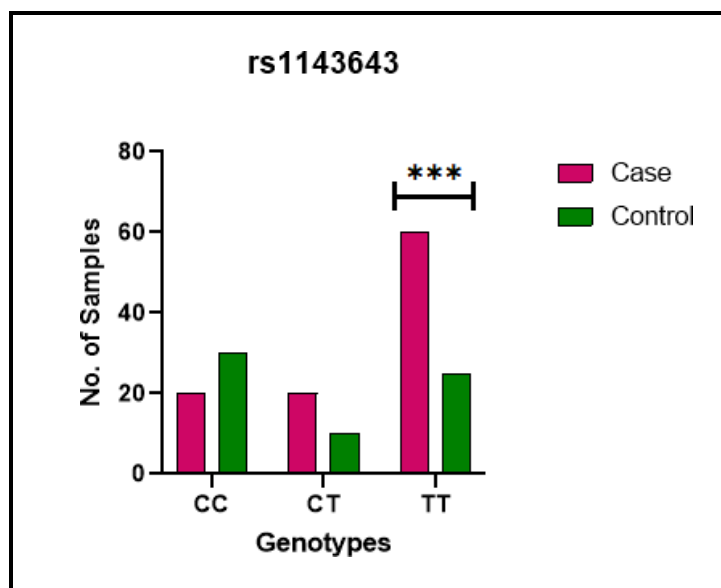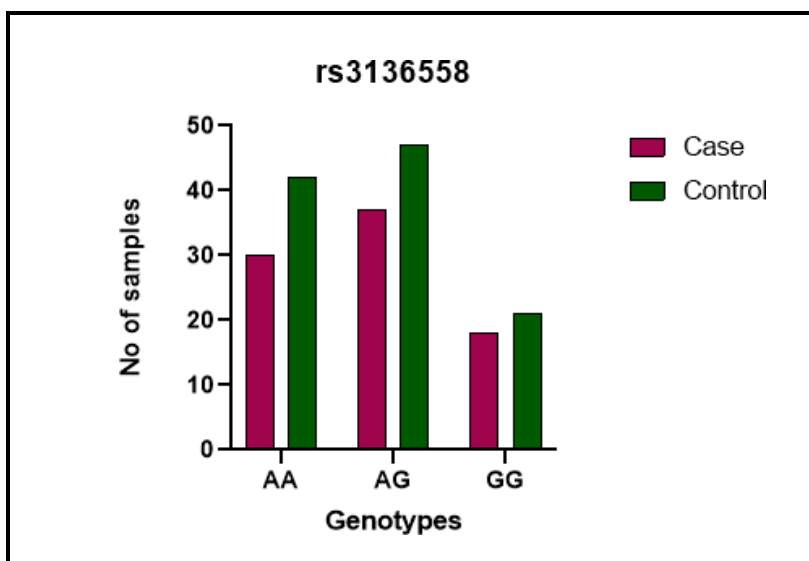
Fig 4.9: Distribution of study subjects across different genotypes for SNP-4: Cases are indicated for RA patients and controls are indicated for healthy individuals. The genotype does not show any significant association of SNP-4 with RA in observed Pakistani population

Table 4.10: Allele and genotype frequencies calculated for study subjects of SNP-4

Cases	Allele frequencies (%)		Genotype frequencies (%)		
RA patients (n=100)	A	G	AA	AG	GG
	60.35	39.65	45.50	32.64	21.86
Controls (n=40)	70.50	29.5	50.86	45.64	23.5

Table 4.11: Hardy-Weinberg equilibrium (HWE) calculations for test population of SNP-4:

	Observed genotype counts			Observed allele counts (%)		χ^2	p-value	Df
	AA	AG	GG	A	G			
RA patients(cases)	37	42	21	60.35	39.65	0.369	0.83	2
Healthy controls	10	14	6	70.50	29.50			
Total	47	56	27	65.35	54.54			

Table 4.12 Statistical association between SNP-4 and Rheumatoid arthritis:

Df	**p-value**	**Association**
2	>0.05	Not significant

CHAPTER #5   DISCUSSION

Discussion

Rheumatoid arthritis (RA) is a chronic disease that mostly affects the synovial joints, affects up to 1% of persons globally. Chronic joint inflammation and progressive joint deterioration are symptoms of RA. (Y. H. Lee et al., 2009). Prostaglandin and metalloproteases are stimulated by IL-1β, which causes bone resorption and joint breakdown. The course of joint injury and plasma and synovial IL-1β levels have been favorably associated. In RA patients, IL-1β receptor inhibition has demonstrated positive therapeutic efficacy (Luotola, 2022).

SNPs are a third-generation molecular marker. SNPs have two basic purposes. First, they directly affect gene transcription, translation, or protein function, which results in a disease or phenotype and SNP may be a genetic marker that is directly linked to a phenotypic or a disease. Previous studies have connected the interleukin cytokine family members interleukin-1 (IL-1), as well as IL-6 (IL-5) and IL-9 (IL-9) to RA (You et al., 2007).

It has been established that cytokines are crucial to the development of RA. Interleukin-1 (IL-1) is one of them and is regarded to be a major mediator of joint degeneration, while IL-1Ra was found to be benefactory in both human and animal clinical investigations. A minimum of three genes, IL-1A, IL-1B, and IL-1RN, which respectively code for IL-1α, IL-1β, and IL-1R antagonist, are grouped on human chromosome 2q14 (You et al., 2007).

Cytokine gene have several variants which have been connected to severity of disease in several chronic as well as progressive inflammatory diseases, including erosive RA. In this study, we investigated possible connections between joint degeneration in RA and interleukin-1 (IL-1) A, IL-1 B (position511), IL-1 B (3954), IL-1RN, interleukin-4 VNTR, and interleukin-4 receptor (IL-4R). Early periarticular juvenile rheumatoid arthritis is associated with the single nucleotide polymorphism (snip) IL-1A in exon V of IL-1α, which is in 100% linkage with the promoter polymorphism IL-1β (889) (Noack & Miossec, 2017b).

TNF and IL-1β are active. The IL-1α and IL-1β proteins are considered the most researched of the IL-1 proteins. By attaching to the IL-1 receptor, they operate like agonist or activator molecules, activating target cells. A natural competitive antagonist as well as transcriptional and post-translational processes control the production and upregulation of IL-1α and IL-1β (Zhao et al., 2021b).

IL-1 levels have been demonstrated to be markedly increased in RA in the past, and this elevation is believed to be related to polymorphisms within the IL-1β genes. Considering their functional significance and chromosomal location as potential diagnostic indicators of RA, polymorphisms within IL-1A, IL-1β, and IL-1RN (Otón & Carmona, 2019).

The IL-1β +3953 C/T polymorphism has been demonstrated to improve IL-1β transcriptional activation and may be linked to higher plasma levels of IL-1β. It is yet unknown, nevertheless, whether the correlation between RA susceptibility and the IL-1β polymorphisms -511 C/T and +3953 C/T is the result of a causative association or a linkage disequilibrium with the actual disease-causing polymorphism (Young & Koduri, 2007).

Previous studies have shown that SNPs of IL-1β are significantly correlated with RA. We examined the relationship between IL polymorphisms and RA susceptibility in this study which was correctly conducted using sound methodologies. Combining data from published studies, we evaluated genetic correlations. In this study, association of four common polymorphisms of IL-1β gene was observed with RA in Pakistani population. The IL-1β gene's SNPs may have a great effect on the disease's distinctive clinical symptoms because these polymorphisms can change the cytokine's level in the body. The amount of the harm done by the effector cell in the inflamed synovium and rheumatoid joints can then be determined by these altered levels.

IL-1β is a key cytokine involved in the inflammatory pathways that ultimately lead to the inflammation and subsequent destruction of the joints in RA. However, polymorphisms show any significant association to the disease. The results of this study and previously carried out studies in relation to RA indicate that other key cytokines, that regulate the levels of IL-1β, are genetically linked to RA. The polymorphisms located in the regulatory regions of these cytokines, must therefore be investigated, for any possible association with the RA.

A total of four SNPs of IL-1β were selected and observed in Pakistani patients. These polymorphisms were examined in the Pakistani population using allele-specific PCR, and the results were statistically examined to see if there was any evidence that these polymorphisms were significantly associated with RA.

Two of the polymorphisms (rs2853550 and rs1143643) in intergenic and intronic regions IL-1β gene showed a highly significant association of RA in Pakistani population. Other two SNPs (rs16944 and rs3136558), indicated no significant difference in the distribution of the observed genotypes in RA patients and healthy individuals. So, our finding focused on observation of rs2853550 and rs1143643 polymorphisms of IL-1β which are associated with progression of RA in Pakistani population as significant results were obtained.

Conclusion:

rs2853550 (A/G) and **rs1143643 (C/T)** genotypes depicts a strong correlation of IL-1β gene in RA population. The crosstalk between these genotypes suggests that the presence of these genotypes play a main role in etiology of RA. The allelic distribution of both SNPs shows that there is significant association of these SNPs of IL-1β gene with RA.

However, rs16944 and rs3136558 polymorphisms of IL-1β gene does not provide a significant association of these SNPs with RA in Pakistani population. The allelic distribution of both SNPs shows that there is no correlation of association of these SNPs with RA and these findings need to replicate in larger population to be authenticated.

Future Aspects

This project will be helpful in dealing with various health related issues worldwide and in Pakistan. The results achieved shall help in investigating different therapeutic approaches and in identifying novel targets for the cure of Rheumatoid arthritis and different autoimmune disorders hence will contribute in some way to the disease management.

Sequencing can also be carried out to possibly identify other variations which may correlate with these SNPs hence would provide more clear function. These SNPs in intronic and intergenic regions are helpful in exploring the mechanism behind the disease progression in RA which was found in this study.

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71